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STUDIES ON ADENOSINE AND CALCIUM ANTAGONISTS IN RAT BRAIN

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Thesis submitted for the degree of Doctor of Philosophy  
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### ABSTRACT

Adenosine is an endogenous neuromodulator that can impose an inhibitory action on synaptic transmission in the peripheral and central nervous system. In the central nervous system the mechanism by which adenosine acts is unclear. One proposed mechanism is by an action at presynaptic calcium channels to reduce calcium influx and subsequent neurotransmitter release.

Three approaches were used to study interactions between adenosine and calcium related events. A neurochemical approach was used to determine the action of adenosine on presynaptic calcium uptake in central nervous tissue. Electrophysiological techniques were employed in the hippocampal slice preparation to study a) the interactions between calcium channel blockers and adenosine compounds and b) the effect of magnesium free ACSF on the inhibition of synaptic transmission by adenosine.

Determination of the uptake of radiocalcium into rat brain synaptosomes across potassium activated voltage dependent calcium channels demonstrated that adenosine compounds could impose a small but significant inhibition on calcium uptake. A similar action was demonstrated on calcium uptake into hippocampal slices, an action that was effectively antagonized by the adenosine antagonist 8-phenyltheophylline

The dihydropyridine class of calcium channel blockers do not affect synaptic transmission in the hippocampal slice, but they do affect the action of adenosine and adenosine analogues. Nifedipine and BayK 8644, a dihydropyridine calcium channel blocker and activator respectively, both act to increase the inhibition of synaptic transmission by adenosine while attenuating the inhibitory action of the adenosine analogues. This interaction appears to involve an effect at both the adenosine receptor and the nucleoside transport system.

In the hippocampal slice the inhibitory action of adenosine on the synaptically evoked population potential was markedly attenuated, and in some cases totally abolished, when magnesium ions were omitted from the artificial cerebrospinal fluid bathing the slice. The proposed explanation for this effect is that activation of NMDA receptor associated channels directly reduces the inhibitory action of adenosine on synaptic transmission.

The results of this study into the action of adenosine on calcium channel associated actions confirm the ability of adenosine to reduce presynaptic calcium influx. It also highlights an interaction between adenosine and the dihydropyridines and provides evidence for an interaction between adenosine inhibition of synaptic transmission and activation of NMDA receptor operated calcium channels.

Abbreviations

LTP	Long term potentiation
VSCC	Voltage sensitive calcium channel
CNS	Central Nervous System
PNF	Peripheral Nervous System
AHP	Afterhyperpolarization
CSF	Cerebrospinal fluid
ACSF	Artificial cerebrospinal fluid
$[K^+]_i$	Intracellular potassium concentration
$[Na^+]_i$	Intracellular sodium concentration
TTX	Tetrodotoxin
TEA	Tetraethylammonium
EDTA	Ethylenediaminetetraacetic acid
DHP	Dihydropyridine
NMDA	N-methyl D-aspartate
2-AP5	2-amino-5-phosphonopentanoic acid
2-AP7	2-amino-7-phosphonoheptanoic acid
R-PIA	R(-) N <sup>6</sup> -(2-phenylisopropyl)adenosine
NECA	5'-N-ethylcarboxamidoadenosine
CHA	N <sup>6</sup> -cyclohexyladenosine
CPA	N <sup>6</sup> -cyclopentyladenosine
DPX	1,3-diethyl-8-phenylxanthine
8PT	8-phenyltheophylline
NBI	Nitrobenzylthioinosine
ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
cAMP	cyclic Adenosine monophosphate
MK801	5-methyl-10,11-dihydro-5H-dibenzocyclohepten-5,10-imine

### Publications

Some of the material presented in this thesis has been published as follows.

- J.T.Bartrup and T.W.Stone (1988). Adenosine inhibition of rat population potentials is magnesium dependent. Br.J.Pharmacol. 94 376p
- J.T.Bartrup and T.W.Stone (1988). Presynaptic actions of adenosine are magnesium dependent. Neuropharmacology 27(7) pp761-763.
- J.T.Bartrup and T.W.Stone (1988). Interactions of adenosine and magnesium on rat hippocampal slices. Brain Res. 463 pp374-379.
- J.T.Bartrup and T.W.Stone (1988). Electrophysiological evidence that adenosine requires magnesium for its interaction at the A1 adenosine receptor. Soc. Neurosci. Abs 14 325.1
- J.T.Bartrup, M.G.Wyllie and T.W.Stone (1988). Adenosine inhibits calcium uptake into rat brain synaptosomes. Br.J.Pharmacol 96 315p.
- J.T.Bartrup and T.W.Stone. (1989) Adenosine inhibits calcium influx into rat brain. In Adenosine receptors in the nervous system. Ed. J.A.Ribeiro. Taylor and Francis.
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# INTRODUCTION

## INTRODUCTION

The field of purine research is a relatively new and complex area that still requires answers to many basic questions. It is now firmly established that adenosine and adenine nucleotides, such as ATP (adenosine 5'-triphosphate), play an important neuromodulatory role in both the peripheral and central nervous systems. What is not clear is the mechanism, or mechanisms by which adenosine exerts its effect on neuronal excitability. This thesis will look at one possible method by which adenosine may act, namely as a calcium channel "regulator", and the interactions of adenosine with other calcium channel blockers, in particular the dihydropyridines, and other divalent cations, notably magnesium.

### Adenosine - an historical overview.

The fact that adenosine or ATP could affect the activity of the body, in particular the heart, was known as far back as 1929 (Drury and Szent-Gyorgyi, 1929). In 1954, Holton and Holton suggested that ATP could be the transmitter released at peripheral and central terminals of sensory fibres after showing that ATP was the vasodilatory substance present in dried, powdered dorsal and ventral spinal root preparations. However, as an excitatory action was being looked for, this idea did not take off. The current interest in adenosine and ATP began with the realization of their potent inhibitory properties on neurones. In the peripheral nervous system the action of ATP as a specific neurotransmitter released from purinergic nerves was put forward by Burnstock (1972).

In the CNS, Phillis et al. (1974) demonstrated the potent depressant effects of iontophoretically applied adenosine in various regions of the brain. Around the same time, Sattin and Rall (1970) were studying the biochemical actions of adenosine and showed that adenosine could stimulate adenosine 3'5'-cyclic monophosphate (cAMP) formation in brain slices, while theophylline and caffeine could antagonize this response. However, research into adenosine as a possible neuromodulator or neurotransmitter was slow until specific binding techniques had been developed that demonstrated distinct extracellular sites for adenosine.

### Adenosine receptors

It subsequently became apparent that adenosine receptors could be classified into subtypes primarily characterized by their ability to affect adenylate cyclase activity. (Londos and Wolff, 1977; Van Calcar et al., 1978, 1979). Thus  $A_1$  receptors are high affinity receptors that reduce the activity of adenylate cyclase and preferentially bind the adenosine analogues R-PIA (R-phenylisopropyladenosine) and CHA (cyclohexyladenosine).  $A_2$  receptors are low affinity receptors that stimulate adenylate cyclase activity and preferentially bind NECA (5'-N-ethylcarboxamidoadenosine). These receptors are situated extracellularly and are antagonized by theophylline and caffeine. The P "receptor" is located intracellularly, has a low affinity, inhibits adenylate cyclase and binds dideoxyadenosine. However, as no evidence has so far been provided for a functional effect of this binding site, it is usually referred to as the P site

(Stone, 1989). More recently further subdivisions have been proposed: the  $A_3$  receptor (Ribeiro and Sebastiao, 1986) that acts independently of adenylate cyclase and inhibits synaptic transmission, possibly by acting directly on calcium channels. The  $A_2$  receptor has also been subdivided into  $A_{2a}$  and  $A_{2b}$  (Bruns et al., 1986),  $A_{2a}$  being a high affinity receptor localized in striatum, and  $A_{2b}$  a low affinity receptor widespread throughout the brain.

The initial observation of adenosine receptor coupling to adenylate cyclase was observed in cultured brain cells (Van Calker et al., 1978, 1979) and fat cells (Londos et al., 1977). However, in the CNS, the association between cAMP activation and neurotransmitter release remains uncertain. Elevation of cAMP levels attributed to  $A_2$  receptor binding has been shown in brain slices (Daly et al., 1983; Fredholm et al., 1982), but inhibition of cAMP accumulation, representing an  $A_1$  receptor action, is difficult to demonstrate. cAMP levels have to be elevated by the use of forskolin or another adenylate cyclase activator, before a decrease by adenosine analogues can be demonstrated (Fredholm et al., 1983; Dunwiddie and Fredholm, 1989). In the case of Daly et al. (1983),  $A_1$  adenosine receptor mediated depression of cAMP was only observed in broken brain tissue membranes, not in more "intact" brain slices.

In parallel biochemical and electrophysiological studies looking at cAMP levels and synaptic depression, R-PIA was shown to be about 40 times more potent at depressing evoked orthodromic synaptic potentials in the hippocampus than at



increasing cAMP levels. (Reddington & Schubert, 1979)

Similarly, evidence that changes in cAMP levels directly affect neurotransmitter release is also weak and unclear. Increases in presynaptic cAMP levels have been shown to both increase (Langley and Weiner, 1978) and decrease neurotransmitter release (Silinsky et al., 1987). Addition of forskolin, which directly activates adenylate cyclase, can also increase (Von Uexkull, 1987) or decrease (Ebstein and Daly, 1982) neurotransmitter release. Consequently, it is not possible to establish a direct cause and effect relationship between the action of adenosine on adenylate cyclase activity, and an increase or decrease in neuronal excitability or neurotransmitter release.

The association of adenosine  $A_1$  and  $A_2$  receptors with adenylate cyclase, and other possible functional sites, is thought to be linked via G-proteins (GTP binding "N" protein). Trussel and Jackson (1987) found that cultured hippocampal and striatal cells responded to adenosine by activating a potassium channel in patch clamp studies. Addition of GTP to cells that had been inactivated by perfusion with saline, restored the action of adenosine. Addition of pertussis toxin, which inactivates certain toxin sensitive G-proteins by ADP ribosylation of the protein, inhibiting their binding to adenylate cyclase, abolished the adenosine activation of potassium channels. Pertussis toxin has also been shown to reduce the inhibitory effect of adenosine analogues on glutamate release from cultured cerebellar cells. (Dolphin and Prestwich, 1985).

There is also evidence that regulation of the potassium channel by adenosine may not involve adenylate cyclase. In the study by Trussel and Jackson (1987) stimulation of adenylate cyclase by forskolin, or addition of cAMP, failed to influence the action of adenosine on the potassium channels. Gross et al. (1989), using voltage clamp techniques to study the inhibitory properties of 2-chloroadenosine on N-calcium channels in mouse dorsal root ganglion cells, showed that treatment with pertussis toxin abolished the inhibition, but forskolin had no effect indicating that adenylate cyclase was not involved. It is also known that proteins exist which are very similar to the  $G_i$  inhibitory protein, but are not linked to adenylate cyclase (Bokoch and Gilman, 1984). It may be this protein that links directly to other possible sites of action for adenosine, such as potassium or calcium channels.

At present adenosine is proposed to act via three mechanisms: 1) activation of potassium channels to hyperpolarize the postsynaptic membrane. 2) inhibition of presynaptic calcium influx to reduce neurotransmitter release. 3) increase or decrease in adenylate cyclase activity. The last mentioned may control the potassium and calcium channels or may act via secondary intracellular mechanisms to reduce neurotransmitter release (Silinsky, 1986b). These will be discussed later. However, at the receptor level, the biochemical route by which the adenosine receptor interacts with the effector mechanism is still unclear. Fredholm and Dunwiddie (1988) have postulated three possible systems:

1) Three subtypes of  $A_1$  receptor linked to the different effector mechanisms via different G-proteins. 2) A single  $A_1$  receptor that can interact with three different G-proteins. 3) A single receptor linked to a single G-protein that can interact and activate all 3 effector systems. The second system is the most compatible with the current data as there is little evidence for multiple  $A_1$  receptor subtypes while the existence of pertussis toxin sensitive and insensitive G-proteins does not favour the third model.

#### Adenosine release.

The purines released from nervous tissue are thought to be largely in the form of adenosine, together with its breakdown products of inosine and hypoxanthine. Studies have been made of the purine overflow from perfused tissue by liquid chromatography (Fredholm and Vernet, 1979). By adding adenosine transport inhibitors, 5'-nucleotidase and adenosine deaminase to modify the production of the various purines (adenosine, ATP, ADP, AMP) in the effluent, it was possible to estimate the levels of the various purines released, adenosine being in the highest concentration. Similar studies using [ $^3$ H]adenosine also showed adenosine to be the major purine released (Bender et al., 1981).

Adenosine efflux from brain slices can be elicited by electrical stimulation, increasing levels by up to 250 times (Pull and McIlwain, 1973). Studies using focal stimuli to elicit overflow of [ $^3$ H]adenosine in hippocampal slices have demonstrated enhanced efflux when perforant path fibres from the entorhinal cortex to the dentate gyrus granule cells are

stimulated, but not when CA1 pyramidal cells are stimulated antidromically via the fimbria (Lee et al., 1982). This indicates that presynaptic terminals need to be activated to initiate release, but whether this involves direct release of adenosine from presynaptic sites, or postsynaptic release as a result of neurotransmitter action, is not clear. The potential release of adenosine from glial cells has also been studied. Lewin and Bleck (1979) used cultured astrocytoma cells to study the uptake and efflux of adenosine. [ $^{14}\text{C}$ ]adenosine was rapidly taken up by the cells, but cells preloaded with [ $^{14}\text{C}$ ]adenine released inosine and hypoxanthine but very little adenosine, suggesting glial cells are sites of adenosine uptake but not release.

The release of ATP has also been shown endogenously from CNS tissue, both in vitro (Pull and McIlwain, 1972; White, 1978) and in vivo (Schubert et al., 1976). It is possible that the released ATP may be broken down to adenosine by the 5'-nucleotidase known to be present extracellularly. However, inhibition of this enzyme does not markedly reduce the levels of adenosine present in the extracellular fluid (Daval and Barberis, 1981) indicating that extracellular conversion of ATP to adenosine is not the major source of adenosine. Other studies, however, have shown a significant proportion of purines released to be in the form of adenine nucleotides, particularly in in vivo studies. (Jhamandas and Dumbrille, 1980).

The release and metabolism of adenosine in the CNS is controlled to give a constant basal concentration of

endogenous adenosine. Measurement of endogenous adenosine levels is difficult, as changes are very rapid, particularly upon death of the animal. One technique employed by Zellerstrom et al. (1982) was to use a hollow dialysis tubing fibre to take cerebrospinal fluid (CSF) samples from the brain of anaesthetized and conscious rats.

Concentrations ranged from 1 to 2 $\mu$ M. In in vitro studies, Fredholm et al. (1984) equilibrated brain slices in a small volume of artificial CSF (ACSF) and obtained final fluid adenosine concentrations of 0.5-1 $\mu$ M. This indicated that the endogenous extracellular adenosine concentration, and adenosine levels released by brain slices are similar.

#### Adenosine uptake

Endogenous adenosine is inactivated by two methods. Adenosine uptake mechanisms are rapid and efficient systems ( $K_m=1\mu$ M, Bender et al., 1981) which suggests they are the predominant method by which endogenous levels are regulated. Adenosine is taken up and appears to be converted back into ATP intracellularly (Bender et al., 1981). The second method is via breakdown of adenosine to inosine by adenosine deaminase ( $K_m=6-60\mu$ M, Arch and Newsholme, 1979). This does not appear to play a key role, as addition of adenosine deaminase inhibitors has very little effect on depression of hippocampal excitability, and levels of adenosine or cAMP are not affected by adenosine deaminase inhibitors, unlike uptake inhibitors that markedly increase levels (Fredholm et al., 1982; Davies et al., 1982).

### Localization of adenosine action

The classification of adenosine as a neurotransmitter has not been possible due to the inability to localize its effects to a specific "adenosine synapse". A number of studies have looked at the distribution of adenosine receptors in the rat brain by using autoradiographic studies of CHA binding. Adenosine receptor sites were most notable in the cerebellar cortex, superior colliculus, neocortex, striatum and hippocampus (Lewis et al., 1981; Goodman and Snyder, 1982). Adenosine receptor density is particularly rich in the hippocampus and is higher in the CA1 compared to the CA3 and dentate gyrus (Murray and Cheney, 1982).

Lesioning of specific neuronal pathways has been used to determine the localization of adenosine receptors. Goodman et al. (1983), looking at the effect of cortical ablation on CHA binding in the striatum, were unable to show any change in receptor number. Similarly, Lloyd and Stone (1985) showed that lesions of the nigrostriatal pathway had no effect on CHA binding in the striatum. Also, kainic acid lesions of striatal cell bodies produced only a 30% decrease in CHA binding density. These results indicate that adenosine receptors are not confined solely to pre- or postsynaptic sites, or to specific neurones in a particular brain region.

Studies on the rat hippocampus have shown the density of A<sub>1</sub> receptors to be differentially distributed through the CA1 region of the hippocampus, with a greater density in the dorsal region compared to ventral (Lee et al., 1983).

Autoradiographic and lesion studies have shown that, within the CA1 region, adenosine receptors appear to be present on presynaptic axon terminals from CA3 cells and on postsynaptic dendrites and cell bodies of the CA1 pyramidal cells (Tetzlaff et al., 1987; Deckert and Jorgensen, 1988; Onodera and Kogure, 1988).

Determination of the sites of activity of adenosine deaminase and the localization of adenosine uptake sites have also been made, but these do not correlate particularly well with the receptor distribution. Bisslerbe et al. (1985), using rat brain to study the binding of nitrobenzylthioinosine (NBI), an antagonist at the adenosine uptake site, showed high densities of uptake sites in the superior colliculus, thalamus, striatum, nucleus tractus solitarius and geniculate nuclei. However, relatively little NBI binding was observed in the hippocampus or cerebellum, regions rich in adenosine receptors. The use of dipyrindamole, another adenosine uptake blocker, showed a slightly different distribution to that shown by NBI, suggesting a heterogeneous population of adenosine uptake sites. However, the dipyrindamole binding site distribution also was not comparable with the adenosine receptor distribution (Bisslerbe et al., 1986, Deckert et al., 1988).

Adenosine deaminase immunoreactivity studies show a good correlation with the distribution of NBI binding sites (Nagy et al., 1985). An extensive plexus of adenosine deaminase containing neurones have been shown in the basal hypothalamus (Nagy et al., 1984), an area largely devoid of

adenosine receptors (Lewis et al., 1981; Goodman and Snyder, 1982). A possible explanation for this particular lack of correlation between receptor density and adenosine deaminase activity, put forward by Stone (1989), is that the hypothalamic nuclei require protection from high levels of blood adenosine that occur during times of hypoxia or increased neuronal activity.

The localization of adenosine itself has also been studied using immunocytochemical techniques (Braas et al., 1986). This has shown adenosine activity to be highest in the pyramidal layers of the hippocampus and dentate gyrus, thalamic nuclei, amygdala, hypothalamus and olfactory cortex. The activity was observed only on neuronal cells, not glia.

The inability to correlate the distribution of adenosine receptors solely to synaptic terminals or with adenosine uptake sites means that adenosine cannot be classified as a classical neurotransmitter. However, adenosine has a definite action on other neurotransmitters, largely inhibiting their effect, and is released endogenously. This release may be as a cotransmitter along with other neurotransmitters from different nerve terminals (Burnstock, 1976). Thus, adenosine can be classified as a neuromodulator at least until such time as a more specific role, or roles, can be ascribed to its presence in the CNS.



### Functional effects of adenosine

Electrophysiological experiments have characterized the inhibitory properties of adenosine. Recordings of neuronal firing have demonstrated a marked depressant action of adenosine in virtually all brain regions tested including cerebral cortex, hippocampus, thalamus, cerebellum and superior colliculus. (Phillis et al., 1974; Phillis and Kostopoulus, 1975). This depressant action is thought to be mediated by reducing presynaptic neurotransmitter release, as adenosine has been shown to depress the release of the majority of established neurotransmitters in the brain including acetylcholine (Vizi and Knoll, 1976; Murray et al., 1982), 5HT (Harms et al., 1979), noradrenaline (Ebstein and Daly, 1982; Fredholm et al., 1983), dopamine (Michaelis et al., 1979), GABA (Hollins and Stone, 1980) and glutamate (Dolphin and Archer, 1983; Corradetti et al., 1984).

Studies of excitatory synaptic transmission in neuronal structures that lend themselves to such studies, such as olfactory cortex (Okada and Kuroda, 1980) and hippocampus (Schubert and Mitzdorf, 1979; Dunwiddie and Hoffer, 1980), have shown that adenosine can reduce the size of evoked synaptic potentials via an adenosine receptor, since theophylline and related xanthine analogues can antagonize this effect. The inhibition is apparent as a decrease in the size of the excitatory postsynaptic potential (EPSP) but adenosine has no effect on the presynaptic fibre volley. This indicates that adenosine is acting at the synapse to reduce synaptic transmission. Intracellular recordings have

confirmed that EPSPs, and IPSPs, are significantly reduced by adenosine (Segal, 1982; Proctor and Dunwiddie, 1983).

Adenosine also has inhibitory effects on responses that are non-synaptic. In particular, adenosine is able to block spontaneous neuronal firing induced by lowering the extracellular calcium concentration, which blocks synaptic transmission. In the hippocampal slice, antidromic stimulation of the pyramidal cells in calcium free medium results in secondary spikes, after the initial antidromic spike, that can be blocked by adenosine (Schubert and Lee, 1986). Adenosine also blocks spontaneous firing of neurones in brain slices that have been largely deafferented as a result of the slice preparation e.g locus coeruleus and cerebellum. It is thought that this non-synaptic action of adenosine is elicited by hyperpolarization of the postsynaptic membrane via potassium channels. Studies of single hippocampal pyramidal cell ion currents (Haas and Green, 1984) have shown a hyperpolarization of the postsynaptic cell together with an enhancement of the after hyperpolarization (AHP) that follows the action potential. In cells that had been pretreated with tetrodotoxin (TTX) to block sodium channels, and barium to enhance calcium channel flux and antagonize  $I_{BK}$  and delayed rectifier potassium currents, a depolarizing current pulse elicited repetitive slow calcium spikes that were superimposed upon a long lasting depolarized plateau. Addition of adenosine (50 $\mu$ M) decreased both the amplitude of the calcium spikes and the duration of the depolarized plateau. Input resistance was not affected

by adenosine. Haas and Green (1984) suggested that these results were due to a calcium dependent increase in potassium conductance by adenosine. In cultured mouse striatal neurones, adenosine seemed to activate potassium currents that induced a hyperpolarization. This effect could be blocked by theophylline and was voltage dependent (Trussel and Jackson, 1985). Thus, the action of adenosine is to increase outward potassium conductance, apparent by the increase in the AHP which hyperpolarizes the postsynaptic membrane, as well as reducing the calcium spikes that are thought to originate in the dendritic region. Increased potassium conductance may also occur presynaptically and reduce the release of neurotransmitter from the synapse, or reduce the probability of the synapse firing when stimulated.

Adenosine has been shown to be particularly potent in blocking spontaneous depolarizations induced in brain slices by various methods including the application of GABA antagonists and altering the extracellular ion concentrations. Ault and Wang (1986) studied spontaneous activity, also called epileptiform bursts, induced by the GABA antagonist bicuculline, in the CA3 region of the rat hippocampus. They tried a number of adenosine analogues and found them to be very potent anticonvulsants, adenosine inhibiting the bursts with an  $IC_{50}$  of  $1.5\mu M$ . O'Shaughnessy et al. (1988) also tried a range of analogues on bursts induced in the rat cortical slice by magnesium free ACSF. Again they demonstrated a potent anticonvulsant effect of adenosine,

the  $IC_{50}$  being  $25\mu M$ . Adenosine in low micromolar concentrations can also reduce bursts induced by penicillin (Lee et al., 1984) and low calcium, both evoked (Lee et al., 1984) and spontaneous (Haas et al., 1984). As low calcium largely inhibits synaptic transmission this suggests that adenosine is not acting presynaptically but may be acting by hyperpolarizing the postsynaptic membrane to inhibit the bursts.

There is substantial evidence that adenosine acts to reduce calcium availability for presynaptic neurotransmitter release. This will be discussed briefly here, but a more detailed discussion forms the basis for the introduction to chapter 3.

Electrophysiological studies have demonstrated the ability of adenosine to modify ion current mediated events. Henon and McAfee (1983), using rat superior cervical ganglion, showed a decrease by adenosine of the amplitude and rate of rise of the calcium mediated spike recorded in solutions containing TTX and TEA to block sodium and potassium channels. 2-chloroadenosine had no effect on the neurone when cobalt and high magnesium were added to block calcium channels. In contrast to the work by Haas and Green (1984), a depression of the AHP was observed with adenosine, R-PIA and 2-chloroadenosine. This effect was enhanced by dipyridamole and blocked by theophylline. Henon and McAfee (1983) suggested that these effects are mediated by a direct action of adenosine on calcium conductance mechanisms, as opposed to enhancement of calcium dependent potassium

currents. A similar conclusion was drawn by Proctor and Dunwiddie (1983) as an explanation for their observation of suppression of calcium spikes by adenosine in hippocampal neurones.

Macdonald et al. (1986) used voltage clamp techniques on cultured mouse dorsal root ganglion cells to show that adenosine reduced depolarization evoked calcium conductance without altering the membrane conductance, indicating that a block was imposed directly on the calcium channel rather than being inactivated by an altered membrane charge. Further studies showed this effect to be confined solely to the N-calcium channel, and to be G-protein dependent but independent of adenylate cyclase activity (Gross et al., 1989). These results also imply a direct suppression of calcium movement by adenosine. Direct measurement of calcium flux by using radioisotopes or calcium sensitive dyes have also provided evidence for regulation of calcium movements by adenosine. This is discussed in chapter 3.

Thus, it appears that adenosine may act in a number of ways: a) to directly suppress calcium influx presynaptically, b) indirectly, via calcium movements, to enhance calcium dependent potassium currents, or c) directly to activate potassium currents and induce a hyperpolarization either postsynaptically, or possibly presynaptically, although there is little evidence for this. The fact that the majority of electrophysiological evidence for regulation of calcium currents has been obtained in

peripheral neuronal tissue, while potassium current data is more readily obtainable from central neurones, may indicate that there are different mechanisms of action for adenosine between the peripheral nervous system (PNS) and central nervous system (CNS). Alternatively, this may simply be due to the difficulty of studying presynaptic ion channels in the CNS.

### Behavioural effects of adenosine

Central or systemic administration of adenosine analogues into an animal causes a number of behavioural effects. Reported effects include loss of motor coordination, analgesia, and changes in food intake, sleep, respiration rate and cardiovascular control (Feldberg and Sherwood, 1954; Haulica et al., 1973). Anticonvulsant actions have also been reported.

The most obvious behavioural effect is an overall reduction in activity, the animal appearing lethargic and sedated. With rodents, the animal remains conscious, responds to noxious stimuli with the appropriate behaviour, and does not lose the righting reflex. In cats and dogs, the effect is more intense with periods of full unconsciousness (Feldberg and Sherwood, 1954). Studies of sleep patterns show that adenosine analogues increase the duration of deep sleep and, at lower doses, increase REM sleep (Radulovacki et al., 1983). Haulica et al. (1973) observed increases in endogenous adenosine of up to 70% in rats deprived of sleep, while studies involving REM sleep deprivation in rats showed

an upregulation of  $A_1$  receptors in the brain (Yanik and Radulovacki, 1987). As adenosine analogues do not directly induce sleep in rodents, it is possible that adenosine helps to induce and maintain sleep rather than being a direct cause of sleep.

Cessation of spontaneous motor activity is another prominent behavioural action of adenosine. At relatively high doses, the animals are nearly immobile, although not rigid. The adenosine analogues R-PIA, NECA and 2-chloroadenosine are particularly potent, with R-PIA having an  $EC_{50}$  of 50 $\mu$ g/kg for the depression of locomotive activity in rodents (Dunwiddie and Worth, 1982). Adenosine itself is much less effective, probably due to its rapid metabolism. The behavioural depression is generally antagonized by the methylxanthines (Dunwiddie and Worth, 1982) demonstrating the receptor mediated nature of the analogues. The actual adenosine receptor subtype involved in these effects is not so clear. Barraco et al. (1983) reported a potency order of NECA > R-PIA > 2-chloroadenosine > S-PIA on locomotive depression. Dunwiddie and Worth (1982), however, found 2-chloroadenosine to be more potent. The solubility of the respective compounds, R-PIA being more hydrophobic than NECA, and the site of administration, centrally or systemically, may explain some of the discrepancies. Hydrophobic  $A_1$  agonists would appear more potent if given systemically as they cross the blood brain barrier more easily, whilst hydrophilic  $A_2$  agonists act faster if given intraventricularly as they do not pass into lipid membranes

and therefore travel through the brain faster (Dunwiddie, 1985). This difference is also manifested in brain slice experiments where hydrophobic analogues have longer onset and washout times than the hydrophilic analogues.

Adenosine analogues have also been reported to have analgesic effects in various behavioural tests, such as the hot plate test. (Yarbrough et al., 1981; Crawley et al., 1981). This effect may be exerted via an interaction with the opiate system, although analgesic effects have been shown to be antagonized by adenosine antagonists, but not naloxone (Yarbrough et al., 1981). Agonist profiles suggest the effect may be exerted via  $A_2$  receptors (Delander and Hopkins, 1987). In vivo and in vitro electrophysiological and neurochemical studies support the behavioural evidence for an interaction between adenosine and the opiate system. Morphine at low micromolar concentrations can enhance the release of adenosine from the surface of the cerebral cortex of anaesthetized rats after systemic administration of the opiate (Phillis et al., 1980). This enhanced release has also been shown in brain slices when adenosine release is induced by depolarizing agents (Stone, 1981a; Fredholm and Vernet, 1978).

#### Role of endogenous adenosine

Despite the extensive effects of adenosine in the nervous system, its exact role, or roles are still not clear. One possibility is as a feedback modulator of synaptic transmission. Adenosine may be released by postsynaptic



membranes to regulate the release of neurotransmitters from the afferent nerve terminals. This regulation may occur continually at a basal level, not just during levels of increased excitability, as added adenosine antagonists increase the level of excitability in resting (i.e. non-stimulated) brain slices (Dunwiddie and Hoffer, 1980). Studies by Dunwiddie (1981) involved repetitive stimulation of hippocampal brain slices in the presence of theophylline or adenosine. With theophylline the response to repetitive stimuli decreased rapidly, presumably as the neurotransmitter pools were depleted. With adenosine the initial response was depressed, but the decline in the response with repeated stimuli was antagonized. This suggests that adenosine is inhibiting neurotransmitter release, reducing the rate at which the neurotransmitter pool is depleted. This also provides further evidence for a presynaptic site of action of adenosine.

This regulation of neurotransmitter release may play a role in sleep/waking cycles in normal neuronal activity, although there is little evidence for this other than an increase in adenosine levels during sleep deprivation (Haulica et al., 1973). Adenosine receptor levels have also been shown to fluctuate throughout the day, possibly to regulate sleep (Virus et al., 1984).

Adenosine may also play a part as an endogenous anticonvulsant, terminating the spontaneous neuronal activity that occurs during an epileptic seizure. The evidence for this is quite convincing. During seizures, the

level of adenosine in the brain increases rapidly (Winn et al., 1980; Schrader et al., 1980). Maitre et al. (1974) showed that adenosine could block audiogenic seizures in susceptible mice. Dunwiddie and Worth (1982) compared CHA, R-PIA and 2-chloroadenosine against a number of convulsant drugs and reported a moderate anticonvulsant activity, with partial antagonism by theophylline in rat studies. The effectiveness of the analogues varied depending on the method of seizure induction. CHA and 2-chloroadenosine were effective against picrotoxin convulsions, but had little effect on kainate and leptazol induced bursts. R-PIA was moderately effective against all bursts. A possible  $A_1$  receptor action was postulated due to the high potency of R-PIA compared to S-PIA, but the possibility of  $A_1$  receptor subtypes was also suggested to explain the varying potency of the analogues on different bursts. Murray et al. (1985) reported similar results, with theophylline antagonizing the effect.

Barraco et al., (1984) showed that intraventricular injections of NECA and R-PIA could antagonize amygdaloid-kindled seizures in rats. Intraventricular injections of adenosine and adenosine uptake inhibitors had a dose dependent anticonvulsant effect on kindled seizures in the rat (Dragunow et al., 1985), while Dragunow and Goddard (1984) showed that the adenosine antagonist aminophylline could accelerate amygdaloid kindling. Similarly, use of theophylline and caffeine facilitated the spread and extended the duration of electrically induced seizures (Albertson et al., 1983). This occurred even at low

doses of the antagonists, suggesting there were no secondary effects involved on other neurochemical actions such as inhibition of phosphodiesterase activity (Amer and Kriegbaum, 1975), increased noradrenaline activity (Galloway and Roth, 1983) or decreased dopamine and 5HT activity (Govoni et al., 1984; Corrodi et al., 1972).

Adenosine receptor binding sites in relation to seizures have also been studied. Chronic theophylline treatment caused an upregulation of CHA binding sites, together with an elevation of seizure threshold (Szot et al., 1987). Treatment with leptazol to induce seizures has been associated with a reduction in the  $A_1$  receptor density in rat cerebellum (Wybenga et al., 1981), although potential tissue damage due to the treatment was not assessed as a possible reason for the reduction.

It may also be postulated that a breakdown or reduction in the efficacy of the adenosine metabolic pathway may actually give rise to epileptic seizures. The addition of xanthines to neuronal tissue in vivo can enhance kindled seizures, as described above (Albertson et al., 1983), while the infusion of methyxanthines into animals can induce spontaneous seizures (Chu, 1981). In the hippocampal slice, addition of theophylline or adenosine deaminase can induce spontaneous depolarizations (Ault and Wang, 1986), suggesting a tonic anticonvulsant action of endogenous adenosine. However, studies on the effect of adenosine on kindled seizures in the amygdala show that adenosine has no effect on the threshold of seizures, only their duration and severity (Dragunow et al., 1985). The authors suggest that

adenosine is released in response to seizures to limit their duration, but as it seems to have no effect on the initiation of the seizure, adenosine cannot be said to act in a seizure prevention role. So, the above data provides some evidence that endogenous adenosine may control epileptic seizures. The development of suitable drugs to increase the efficacy of endogenous adenosine may be of particular pharmaceutical value in the prevention and treatment of epilepsy.

A number of neurological disorders are thought to be associated with a disorder of the purinergic system. The inherited condition, Lesch-Nyhan Syndrome, inflicts the sufferer with involuntary movements, as well as mental retardation, self mutilation and hyperuricaemia. The cause of this is a lack of hypoxanthine-guanine phosphoribosyltransferase (HGPRT) activity, an enzyme which salvages excess purine nucleotides, converting them to, among other things, inosine 5'-monophosphate which is a substrate for adenosine synthesis (Fig.2.). Low levels of adenosine are observed in Lesch-Nyhan patients and this may play a part in the neurological problems, particularly the spasticity that affects the patient (Stone, 1981c).

Another hereditary neurological disorder is Gilles de la Tourette's syndrome, characterized by involuntary tics and limb jerks, as well as self-mutilation and involuntary outbursts. This has also been linked to a reduction of HGPRT activity (Van Woert et al., 1977), and some of the symptoms may be a result of reduced adenosine activity.

### Adenosine compounds

The properties of the main adenosine analogues used in the experiments of this thesis are described below. Their structures are shown in Fig.1.

#### Adenosine

Adenosine is the endogenous ligand for adenosine receptors. It is active at both  $A_1$  and  $A_2$  receptors and is readily taken up by the nucleoside transport system. The hemisulphate salt of adenosine was used in the present experiments. This is readily soluble in water but otherwise has identical properties to free base adenosine. Endogenously, adenosine is formed primarily from AMP via the action of 5'-nucleotidase and is inactivated by conversion to inosine by adenosine deaminase (Fig.2.). Adenosine is not a principal product of the de novo synthesis of purine metabolism, but is a metabolite of the nucleotides and can be reconverted to AMP by adenosine kinase (Stone, 1981c). The adenosine molecule is the basic structure for the adenosine analogues, with small changes to the molecule altering the potency and receptor selectivity.

#### 2-chloroadenosine

An analogue of adenosine that, like the other adenosine analogues, is not taken up by the nucleoside transport system (Clanachan et al., 1986). This makes it approximately 10x more potent than adenosine on hippocampal CA1 evoked potentials. 2-chloroadenosine acts at  $A_1$  receptors with some affinity for the  $A_2$  receptor.

N-ethylcarboxamidoadenosine (NECA)

Analogue used as a ligand for the  $A_2$  receptor in binding studies, but has potent inhibitory action on hippocampal cells. If the receptor has a greater affinity for NECA than R-PIA, this is used as a criteria for classification as an  $A_2$  receptor.

R(-)-(phenylisopropyl)adenosine (R-PIA)

A potent adenosine analogue selective for the  $A_1$  receptor. The receptor affinity ratio of R-PIA and its less active S isomer is used to classify  $A_1$  receptors, the ratio being greater for  $A_1$  receptors. This compound is hydrophobic and requires an ethanol or DMSO vehicle. Its hydrophobic nature gives it a long onset and washout time in functional studies.

Cyclohexyladenosine (CHA)

A potent analogue highly selective for the  $A_1$  receptor. The compound is hydrophobic, requires an ethanol or DMSO vehicle and has a long onset and washout time in functional studies.

Cyclopentyladenosine (CPA)

A potent analogue with a slightly higher selectivity than CHA for the  $A_1$  receptor. This compound is also hydrophobic, requires an ethanol or DMSO vehicle and has a long onset and washout time in functional studies.

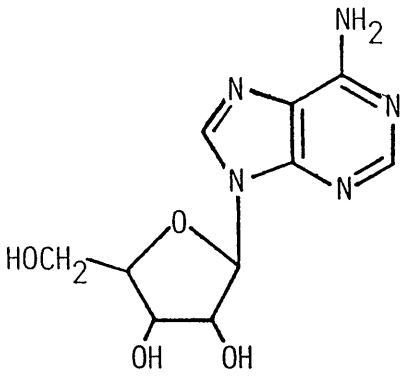
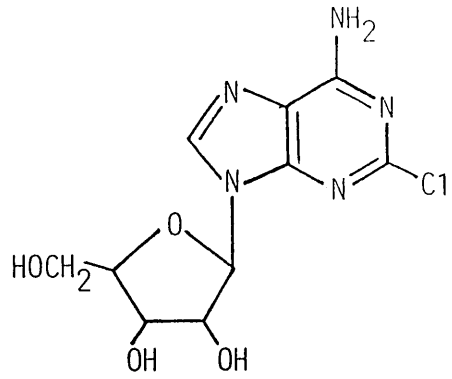
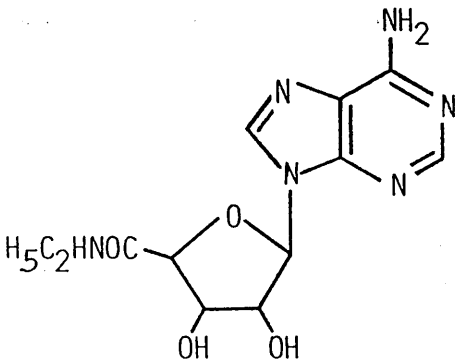
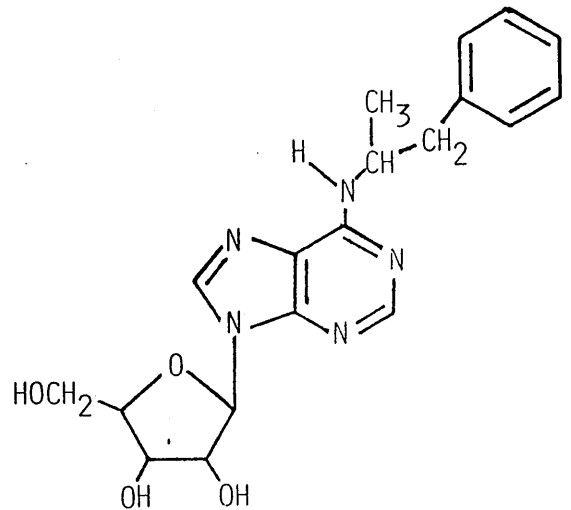
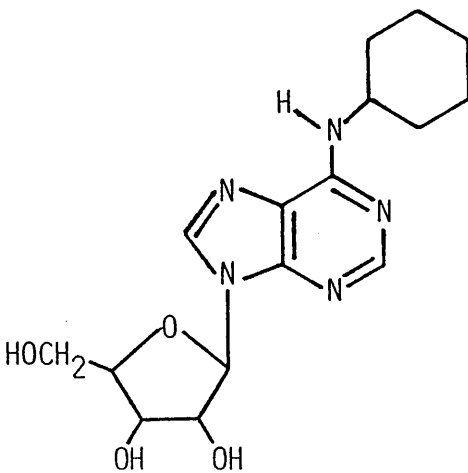
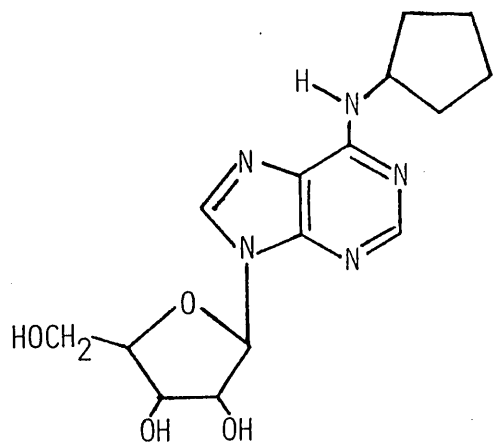
Adenosine2-Chloroadenosine5'-N-Ethylcarboxamide adenosine (NECA)R-Phenylisopropyl adenosine (R-PIA)Cyclohexyladenosine (CHA)Cyclopentyladenosine (CPA)

Fig. 1. Molecular structures of the most frequently used adenosine compounds.

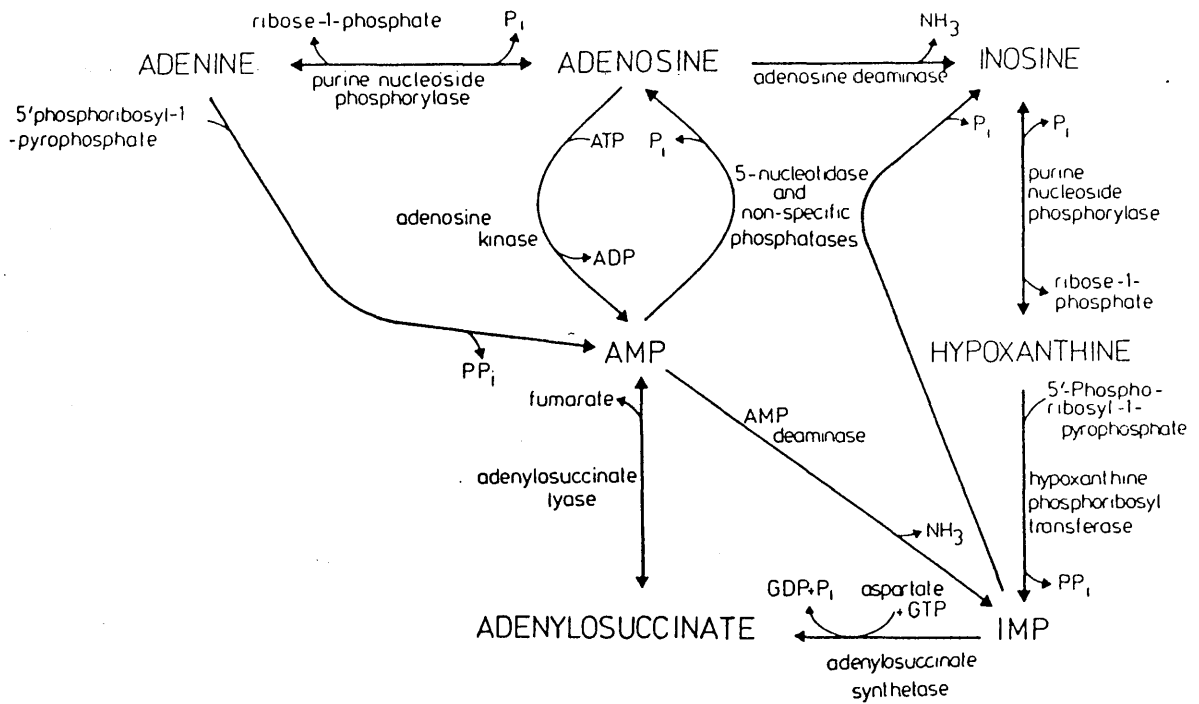


Fig. 2. Biochemical pathways involved in adenosine metabolism. (Reproduced from Arch and Newsholme 1979).



### Calcium channels

The inhibitory action of adenosine in the CNS is postulated to involve an action on neuronal calcium channels, reducing calcium influx. There is also evidence of interactions of adenosine with the dihydropyridine class of calcium channel blockers, which might possibly involve a combined action at neuronal calcium channels. As an introduction to an investigation into these effects, the nature of the different types of calcium channels and calcium channel antagonists will be discussed.

The calcium gradient between the inside and outside of cells is large, extracellular calcium being  $10^{-3}\text{M}$  compared to  $10^{-7}\text{M}$  intracellularly. This gradient is maintained by the relative impermeability of the cell membrane and by active extrusion mechanisms. These include a specific calcium ATPase that actively removes calcium from inside the cell, and a sodium / calcium exchange system that exchanges three or more sodium ions for one calcium ion. Calcium is also stored intracellularly in the smooth endoplasmic reticulum, mitochondria and bound to proteins such as calmodulin. Maintenance of a large calcium gradient is particularly important in excitable tissues such as cardiac and smooth muscle, and neuronal tissue. Depolarization of the cell membrane results in an influx of calcium down the gradient which aids in the activation of the tissue functions. In cardiac cells, calcium ions prolong the action potential to regulate the rate of cardiac contraction. In muscle cells, calcium is required for contraction of the muscle fibre,

while in neuronal tissue, calcium influx facilitates the release of neurotransmitter from the synaptic terminal. Depolarization of the presynaptic terminal opens voltage sensitive calcium channels and the resulting increase in intracellular calcium triggers the release of neurotransmitter by a mechanism that is, as yet, not fully understood. Thus the voltage sensitive calcium channel is a principal site for the regulation of neuronal excitability.

The calcium influx occurring along voltage sensitive calcium channels (VSCC) is one of the two main routes of controlled calcium entry, the other being receptor operated calcium channels. The voltage sensitive calcium channel is a large membrane spanning glycoprotein molecule that allows controlled entry of calcium ions into the cell down the concentration gradient. Part of this molecule can alter to open or close the channel. The probability of this gate being open is dependent on the depolarized state of the membrane and can also be altered by hormones, neurotransmitters and neuromodulators (such as adenosine). It is not known whether separate populations of channels are affected by the various modulators, or whether channels of a similar type can be affected by a variety of receptor and voltage actions. Part of the channel also acts as a selectivity filter, possibly momentarily binding calcium as it passes through to prevent other ions passing. Certain divalent metal ions can bind tightly to this filter and block the channel (Stanfield, 1986).

Studies of cell membrane calcium channels have characterized three subtypes of VSCC distinguishable by their voltage dependencies, selectivity and pharmacology as well as their kinetic signature under single channel recording. Nowycky et al. (1985) used chick dorsal root ganglion cells to study and characterize the calcium channels. Studies of other tissues have found the characteristics to be largely consistent, so the nomenclature is used for VSCCs in most tissues.

The channels have been named T, N and L calcium channels:

T (transient) channels are activated by weak depolarizations ( $>-70\text{mv}$ ), inactivate very rapidly (20-50msecs) and are open only transiently during a period of depolarization. This channel is largely resistant to block by cadmium but not nickel and is insensitive to dihydropyridine calcium channel blockers and  $\omega$ -conotoxin. Because of its rapid inactivation rate it has been postulated that the channel is involved in the control of rhythmic activity, such as heart pacemaker potentials, or bursts of neuronal activity.

L (long opening) channels are activated by a relatively strong depolarization ( $>-10\text{mv}$ ) but remain open for long periods of time ( $>500\text{msecs}$ ) during the depolarization. They are sensitive to block by cadmium but resistant to nickel. The dihydropyridines act principally on this channel while  $\omega$ -conotoxin exerts only a weak block. The L-channel is found in most tissues, but principally in cardiac and smooth

muscle, and is thought to be involved in the action potential and contraction of muscle tissue, and possibly in neuronal action potentials.

N (neither T nor L, or neuronal) channels are activated at relatively strong depolarizations ( $>-30\text{mv}$ ) from a very negative resting membrane potential ( $-120$  to  $-30\text{mv}$ ). They inactivate moderately fast ( $50-80\text{msecs}$ ) but continue to open and close during a depolarization. They are sensitive to block by cadmium and  $\omega$ -conotoxin, but insensitive to the dihydropyridines. They are found predominantly in neuronal tissue and may be located at the synapse to regulate neurotransmitter release.

The above voltage parameters are for the chick dorsal root ganglion. In other tissues these may vary. For example, in rat sympathetic neurones, the inactivation rate for N channels can be around  $500\text{msecs}$ . It is likely that further studies will increase the number of channel classifications. Suszkiew et al. (1989) has already proposed a different calcium channel type for mammalian brain synaptosomes. Also, studies of skeletal muscle show channels that do not fit any of the above classifications.

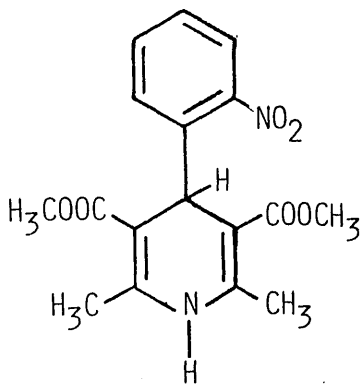
#### Calcium channel blockers.

A range of calcium channel antagonists are available that are principally used in the treatment of smooth muscle and cardiac related problems such as hypertension, angina and cardiac arrhythmias. The principal compounds are: the phenylalkylamines, originally synthesized by Hoechst in

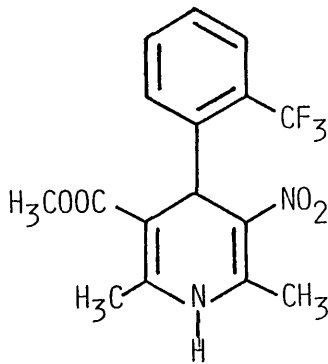
1963, which include verapamil and gallopamil (D600); the benzothiazepines, in particular diltiazem, developed by Tanabe Seiyaku Co., Japan in 1975; and the dihydropyridines (DHP), including nifedipine, nimodipine, nitrendipine, and nisoldipine, as well as the agonist BayK 8644 which were originally developed by Bayer around 1969. The structures of the principal compounds are shown in Fig.3. All three classes of drugs have potent calcium channel blocking properties in smooth and cardiac muscle; verapamil is equally effective in both types of tissue, while the dihydropyridines are more effective in smooth muscle. (Reviews: Van Zwieten, 1982; Fleckenstein, 1983). However, their effects on neuronal calcium channels are much weaker and possibly, in the case of verapamil, may also affect sodium channels (Norris and Bradford, 1985). The main drugs of interest in this study are the dihydropyridines, but the other calcium channel blockers will be briefly discussed to put the significance of their effects into perspective.

The phenylalkylamines, verapamil and its methoxy derivative D600, are well established calcium channel antagonists that have potent inhibitory effects on smooth muscle contractility, (e.g.  $ID_{50}$  on  $K^+$  stimulated contraction of rabbit aorta, 27nM, Schuman et al., 1975). Verapamil is used clinically to treat angina and cardiac arrhythmias. In neuronal tissue the effect is much less potent, requiring micromolar concentrations to have any effect on calcium movement, although Norris and Bradford (1985) were able to show a significant reduction in  $^{45}Ca$  uptake into cortical

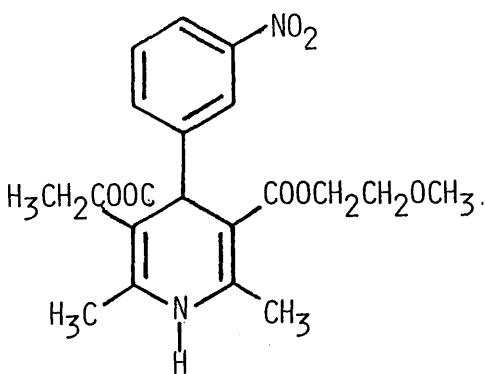
Nifedipine



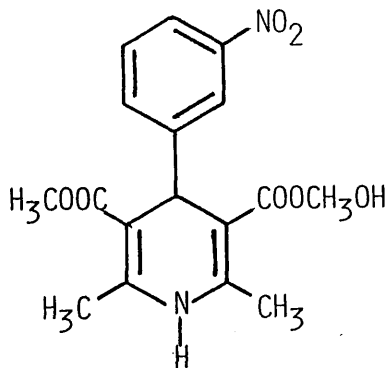
Bay K8644



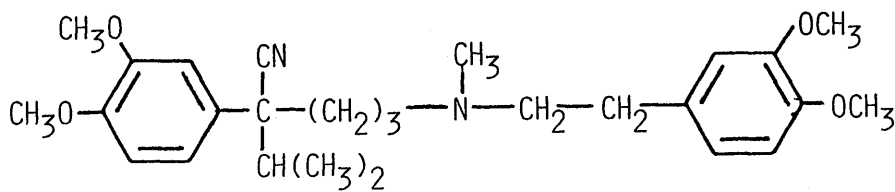
Nimodipine



Nitrendipine



Verapamil



Diltiazem

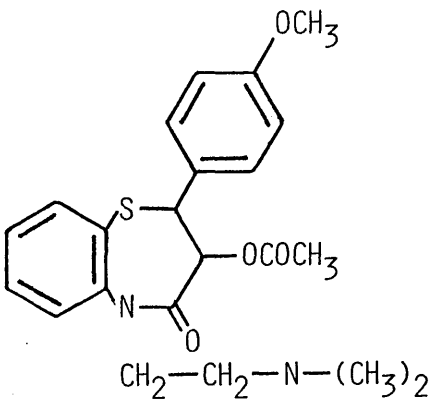


Fig. 3. Molecular structures of the calcium channel antagonists.

synaptosomes by verapamil at 0.5 $\mu$ M. (In a subsequent paper, however, they were unable to show an effect below 25 $\mu$ M, (White and Bradford, 1986)). At high concentrations it is very likely that verapamil is exerting its effect via sodium channels rather than calcium channels (Naschen and Blaustein, 1975; Erdricht et al., 1983). To quote Miller and Freedman (1984), "At concentrations above 10<sup>-6</sup>M, verapamil and D600 will block virtually every biological process known to man."

Diltiazem is also very active in smooth muscle and is used to treat angina. It is thought to bind to the same site as verapamil, although there is some controversy over this. Both diltiazem and verapamil bind to a separate site from the dihydropyridines, but can displace them via allosteric interactions in binding studies (Ehlert et al., 1982). Diltiazem, like verapamil, has a very weak action on neuronal calcium influx (ID<sub>50</sub>=70 $\mu$ M, Wei and Chiang, 1986).

### The dihydropyridines

The binding of dihydropyridines (DHP) to sites in central nervous tissue can be readily shown (Gould et al., 1982; Belleman et al., 1983) and appears to have differing regional distributions, certainly within the rat brain (Skattebol and Triggle, 1987), the highest density being found in the hippocampus and olfactory bulb. However, the binding sites do not appear to correlate with a functional effect of the DHPs. Daniell et al. (1983), studying <sup>45</sup>Ca uptake into rat whole brain synaptosomes, were unable to show any effect of nisoldipine, nimodipine or nitrendipine

on uptake at concentrations from 0.1nM to 10 $\mu$ M. Only at low extracellular calcium concentrations (0.12mM) with strong potassium depolarization (109mM) were they able to show any significant decrease in uptake. Using the same technique Rampe et al. (1984) were unable to show any effect of the DHP calcium channel agonist BayK 8644 on uptake, despite the fact that high affinity [ $^3$ H]nitrendipine binding could be shown that was competitively antagonized by BayK 8644. A similar lack of effect was reported by Massieu and Tepia (1988).

Inhibition of  $^{45}$ Ca influx can be shown by other calcium channel blockers. Wei and Chiang (1985, 1986) showed that flunarizine and D600 could reduce potassium stimulated influx into rat cortical synaptosomes at low micromolar concentrations (flunarizine ID<sub>30</sub> 0.7 $\mu$ M), while nifedipine and nitrendipine were effective only at high concentrations (nifedipine ID<sub>30</sub> 100 $\mu$ M), despite the high affinity binding displayed by [ $^3$ H]nitrendipine. As stated by the authors, this is in contrast to the potent effect of the DHPs in smooth muscle and indicates that the calcium channels are different from those in non-neuronal tissue. Similar results were obtained by Adron Harris et al. (1985).

The above studies have so far been carried out using freshly prepared brain synaptosomes. The use of cultured neuronal cells presents a different picture. Carboni and Wojcik (1985, 1988) used primary cultures of cerebellar granule cells to show a single high affinity binding site for [ $^3$ H]nitrendipine, the binding of which could be



displaced by nitrendipine, nifedipine and BayK 8644 at nanomolar concentrations. However, in this preparation, they were able to show a 40% inhibition of potassium stimulated  $^{45}\text{Ca}$  influx by nifedipine and nitrendipine at  $0.1\mu\text{M}$ , although this was not increased at higher concentrations. BayK 8644 gave a similar increase (30-35%) in calcium influx. The authors concluded that a subclass of DHP sensitive calcium channels were present in the cerebellar cells comprising 40% of the total population. Subsequently, Thayer et al. (1986) showed that cultured cells from different regions of the rodent brain exhibited varying sensitivities of VSCC block by DHPs. Hippocampal cells showed a 79% inhibition with nitrendipine ( $1\mu\text{M}$ ) while striated neurones showed only a 31% inhibition. On the other hand, BayK 8644 showed a doubling of intracellular calcium in all cells studied, including mixed cell cultures. The cells were depolarized by potassium and the concentration of intracellular free calcium was measured using fura 2 and a microspectrofluorimeter. The use of fura 2 means that the source of the calcium cannot be determined, the DHPs may be acting by preventing release from intracellular calcium stores. However, the different sensitivities to the DHPs between brain regions correlates with binding studies and the distribution of rapid calcium uptake sites (Skattebol and Triggle, 1987).

Electrophysiological studies have also demonstrated the action of DHPs in neuronal tissue. Using voltage clamp techniques on hippocampal cells in slices, Docherty and Brown (1986) were able to show that nimodipine was able to

reduce a persistent calcium current (possibly an L channel) by 55% at 2 $\mu$ M. Lower doses gave a correspondingly lower inhibition, but a total block was not possible even at 10 $\mu$ M. A second calcium current, possibly a T or N channel, was also observed, but this was largely insensitive to the DHPs. BayK 8644 (5 $\mu$ M) was able to enhance the persistent current in some cells, although this effect was not constant.

The organic calcium channel blockers affect the flux of calcium through the voltage sensitive calcium channel. It is likely that the dihydropyridine receptor is associated directly with the VSCC and that binding sites for the DHPs also represent the distribution of VSCCs (Miller and Freedman, 1984). The DHP binding site in skeletal muscle has been characterized as a four polypeptide molecule incorporating the calcium channel and sites for binding of the DHPs, verapamil and diltiazem (Campbell et al., 1988). Whether this is the structure for neuronal DHP binding sites is not certain, but possible. The problem of why the high density of DHP binding sites does not correlate with a functional action may possibly be explained by a structural difference between the calcium channel subtypes. The DHPs may be capable of binding to all the VSCCs, but only the DHP sensitive (L) calcium channels respond by closing. Assuming neuronal calcium channels are predominantly N type channels, with only a small proportion of L channels, then only a minimal effect of the dihydropyridines might be expected to be observed in neuronal tissues. Another possibility is a difference in the experimental techniques used to study

calcium channels. Neuronal cells in vivo are subject to multiple inputs from neighbouring excitatory and inhibitory neurones as well as neuromodulatory influences. This largely determines the resting membrane potential and the degree of depolarization upon stimulation of the cell. When these cells are broken up in the preparation of synaptosomes the regulatory inputs are lost and the voltage dependent channels may respond in a different manner to that which occurs in vivo. Primary cultured and clonal neuronal cells, on the other hand, develop largely without any regulatory inputs and the channels in these cells may therefore exhibit different properties to "fresh" cells. The modulated receptor hypothesis (Hondeghe and Katzung, 1984) proposes that certain drugs can only bind to channels when the channel is in an open state. This hypothesis has been applied to the dihydropyridine sensitive calcium channel by Chin (1986) to try and explain the differential sensitivity of calcium channels to the dihydropyridines. Assuming that voltage sensitive calcium channels in isolated synaptosomes and cultured neuronal cells differ in their active state as a result of their developmental environment, this hypothesis may explain why dihydropyridine action is more apparent in clonal cells than in synaptosomes.

#### Behavioural effects of the dihydropyridines

Reports of the psychopharmacological effects of the dihydropyridines in animal behavioural studies are relatively scarce, but there is some evidence that the dihydropyridines do exert an effect in the CNS.

Hoffmeister et al. (1982) compared the effects of nimodipine with a number of neuroleptics and anxiolytics in a wide range of behavioural studies. The nimodipine was administered peripherally in all experiments. In mice, nimodipine impaired the ability of the animal to balance on a rod, and reduced aggressive behaviour. It inhibited seizures induced by pentylenetetrazol, but had no effect on seizures induced by electroconvulsive shock. Nimodipine exhibited no cataleptic effects and did not antagonize catalepsy induced by butyrylperazine in mice or rats. However, it potentiated the cataleptic effect of reserpine in rats, an effect normally only seen with the major tranquillizers. Studies of resting EEG, recorded from leads chronically implanted in a number of brain regions in the cat, showed nimodipine to have no effect on the various EEG patterns of sleep and waking states. However, nimodipine did appear to inhibit the slow sleep EEG elicited by administered 5-hydroxytryptophan (precursor of serotonin) in cats chronically pretreated with parachlorophenylalanine to decrease endogenous monoamine levels, suggesting that nimodipine impairs serotonin formation or action. A subsequent report on the inhibition of flurazepam induced sleep by nifedipine in rats may be the result of a similar action on serotonin by nifedipine (Mendelson et al., 1984). A final positive effect of nimodipine was to reduce amnesia caused by electroshock or hypoxia in rats and mice. The animals were more likely to remember to avoid an area associated with an electric shock if given nimodipine before the amnesic stimuli.

The effects of nimodipine in the behavioural models described above cannot be explained by an effect on the cerebrovasculature, but suggest a direct effect on neuronal functions. However, the effects as a whole are not very significant, particularly in comparison with established psychopharmacologically active drugs. There may be a mild anxiolytic effect, as suggested by suppression of aggressive behaviour, and a possible anticonvulsant action on drug induced seizures. There were, however, no major behavioural effects that could be attributed solely to nimodipine.

The dihydropyridine calcium channel agonist BayK 8644 has been shown to have slightly more pronounced behavioural effects. Bolger et al. (1985), studying the effect of i.p. administered BayK 8644 in mice, reported a marked ataxia together with acute muscle tension; arching of the back and limb extension. This did not appear to be a direct effect on the muscles themselves as the animal could exhibit periods of rapid motor activity if provoked. There was also an increase in auditory sensitivity, with wild running and audiogenic seizures in response to loud noises in some cases. A similar set of behavioural effects could be induced with reserpine, suggesting that BayK 8644 may be acting to decrease the action of a number of neurotransmitters including 5HT, noradrenaline and dopamine. The effect of BayK 8644 could be blocked by the administration of nifedipine. This was not due to an effect of nifedipine on the cerebral vasculature as prazosin, another vasodilator,

had no effect on the action of BayK 8644. Nifedipine on its own had no obvious behavioural effects.

Thus, the dihydropyridines have been shown to have mild behavioural effects in animals that can be ascribed to an action on the CNS as opposed to any peripheral action on the vasculature. BayK 8644 appears to have the most marked effect. However, all the effects could possibly be explained by interactions of the dihydropyridines with endogenous neurotransmitters and neuromodulators, rather than from any direct action of the dihydropyridines via calcium channel actions.

### The hippocampal slice.

Slices of mammalian CNS tissue, maintained in vitro, were originally shown to be physiologically viable by Yamamoto and McIlwain (1966). Subsequently, the hippocampus proved to be a useful brain structure from which stable electrical activity could be recorded over a number of hours (Skrede and Westgaard, 1971; Bliss and Richards, 1971; Yamamoto, 1972; Schwartzkroin, 1975). The hippocampal slice is now used extensively for a wide range of neurochemical and electrophysiological experiments, its use being reported in over 117 publications during 1988 (Medline). Techniques that can be used in conjunction with the hippocampal slice include radiolabelled neurotransmitter release studies and calcium uptake studies. Electrophysiological techniques can be employed to study three principal regions of the hippocampal slice; the CA1, CA3 and dentate gyrus. The pyramidal cells of the CA regions can be activated both orthodromically and antidromically to study pre- and postsynaptic actions of the cells and are also useful for single cell and intracellular studies. The neuronal pathways of the hippocampus readily exhibit spontaneous activity under a variety of conditions; particularly useful as in vitro models of epilepsy.

The hippocampus is organized in layers which fold over in a loop to form the CA (Cornu ammonis) areas 1 to 4, and the dentate gyrus. In the CA1 region, the layers consist of the alveus, which principally contains the axons of the pyramidal cells that pass out of the hippocampus along the

fimbria of the fornix. Below this is the Stratum oriens up through which the pyramidal cell axons pass. Collateral fibres from adjacent pyramidal cell axons travel through this area down to the Stratum radiatum, and small inhibitory basket cells are present that synapse with the pyramidal cells. The next layer, the Stratum pyramidale, contains the cell bodies of the pyramidal cells. These are clearly visible as a dark band which makes positioning of electrodes relatively easy. The Stratum radiatum is below this and contains the apical dendrites of the pyramidal cells together with the Schaffer collaterals which are collateral branches from axons of pyramidal cells in the CA3 region. The apical dendrites pass down into the next layer, the Stratum lacunosum-moleculare, where they branch profusely. The Schaffer collaterals from CA3 pyramidal cells and commissural fibres from the contralateral hippocampus synapse onto these dendrites. A stimulating electrode placed on these collateral and commissural fibres will enable the synapses to be activated. The resulting depolarization of the pyramidal cells can be observed as a characteristic potential that is representative of the level of synaptic transmission and the number of depolarizing pyramidal cells (Andersen et al., 1971). The pyramidal cells, together with their axons and dendritic trees, as well as the Schaffer collaterals and commissural fibres, all lie in parallel such that a transverse slice of the hippocampus will contain an intact section of cells complete with connections to other cells in the parallel plane of the slice.



The advantages of an in vitro brain slice preparation over in vivo brain electrophysiological studies are many. There are no problems with pulse and respiration movements, no anaesthetic problems and no blood pressure and respiration levels to observe and maintain. The preparation is relatively easy and quick with no lengthy pre-experimental surgery to contend with. Positioning of the electrode is easy, with a relative degree of certainty as to its location with respect to specific areas, such as the cell body or synaptic regions. Application of drugs, whether by bath application or iontophoresis, results in a rapid response and wash out. With no input from other brain regions, absence of neurohumoral effects and a limited number of cells it is possible to be fairly certain that a response to a drug or experimental manipulation is from a single population of cells, rather than a vast number of possible interactions with other brain regions.

A number of studies have looked at the viability and integrity of the hippocampal slice. Schurr et al. (1984) carried out a detailed study on the viability of the slice over time. CA1 evoked population potentials reached a stable level after 1-2 hours incubation and remained stable for a minimum of 6 hours (mean  $11.7 \pm 4$ ). Electron microscopic studies showed "healthy" pyramidal cells with large nuclei containing finely dispersed chromatin and a prominent nucleolus. Cytoplasmic components were apparent with ovoid mitochondria, RER, golgi complexes, microtubules and microfilaments. Between 6 and 12 hours there was a general

degeneration of the cells with the appearance of vacuoles in the cytoplasm, "burst" mitochondria, pycnotic nuclei and general swelling of dendrites. The progression of this degeneration was rapid after onset and electrical activity disappeared soon after onset. The duration of the experiments carried out for this thesis did not last longer than 5 hours including preincubation.

Determination of the metabolic stability of the rat hippocampal slice by Whittingham et al. (1984) showed the energy charge (determined by measuring adenylate levels) and ATP/phosphocreatine ratio values to be close to in vivo hippocampal levels. Total levels of ATP, adenylate, creatine and phosphocreatine were about half the in vivo levels, but attempts to improve the metabolic profile by adding adenylate precursors and amino acids have little effect (McIlwain et al., 1951).

The thickness of the slice is an important consideration. A layer of damaged tissue, between 10-100µm thick, is present on either side of the slice (Reid et al., 1988) and in slices thicker than 500µm, there may be a core of anoxic tissue where oxygen is unable to diffuse (Whittingham et al., 1984). A slice thickness of between 400-500µm is therefore preferred.

As with any in vitro preparation there are a number of disadvantages that need to be considered and controlled if possible. Responses elicited from cells may not be typical of the cells when in vivo. Excitatory and inhibitory inputs from other cells have been cut, in particular GABAergic inhibitory neurones which run longitudinally in the hippocampus and are cut when the hippocampus is sliced transversely. Neurohumoral control is absent or abnormal. The slices contain a large number of damaged and degenerating cells as a result of the preparation. These may release factors that affect other cells, including amino acids (Balcar and Johnston, 1975), or partially damaged cells may contribute to the observed response, giving abnormal results. The ACSF used to bathe the tissue may give good, reproducible responses, but whether it provides the tissue with conditions close to those found in vivo is not clear (Whittingham et al., 1984). Although a sample of CSF can be analyzed for its ionic constituents, the ionic concentration in the extracellular space is not easily determined.

If these advantages and limitations are taken into account, and the aims and interpretations of the experiments determined accordingly, then the brain slice can be a very useful and informative experimental preparation.

# CHAPTER 1

## CHAPTER 1

### THE ROLE OF MAGNESIUM IN THE INHIBITORY ACTION OF ADENOSINE.

#### 1.1. INTRODUCTION

The effect of adding adenosine to an in vitro C.N.S. preparation is, in most cases, to depress neuronal excitability. This is in addition to a tonic inhibition of excitability imposed by endogenous levels of adenosine known to be present both in vivo (Zellestrom et al., 1982) and in vitro (Fredholm et al., 1984). The addition of adenosine antagonists, or the removal of endogenous adenosine by adenosine deaminase, results in an increase in excitability that convincingly demonstrates tonic inhibition (Ault and Wang, 1986; Snyder et al., 1981). A reduction in the endogenous concentration of adenosine in vivo might conceivably lead to an increased neuronal excitability that would manifest as convulsions and epileptic seizures. This is the case with experimentally reduced levels of adenosine in vitro (Ault and Wang, 1986), but pathological or hereditary conditions may also lead to epilepsy via a reduction in adenosine efficacy. One possible area that might affect adenosine is the composition of the ions in the cerebrospinal fluid (CSF).

The composition and concentration of ions in the CSF bathing neuronal tissue is very finely balanced. Any alteration can often lead to detrimental changes in neuronal activity. In vitro models of epilepsy have frequently employed changes in extracellular ion concentration to

induce spontaneous neuronal activity. A change in the concentration of most of the ions in the ACSF will alter the excitability of the tissue:

Elevating extracellular potassium to around 8.5mM produces spontaneous depolarizations in the hippocampal slice. This is thought to be due to a reduction in resting potassium currents that alters the balance of inward and outward ion currents, increasing the probability of neuronal firing. There may also be a reduction in GABA mediated IPSPs as a result of an increased intracellular chloride concentration, possibly due to the elevated potassium activating inward KCl pumps or via passive influx. (Korn et al.,1987).

Possibly via a similar mechanism, ACSF in which 60% of the chloride has been replaced by an inert negative ion such as methylsulphate, results in epileptiform bursting after a brief electrical stimuli. This is thought to be due to reduced GABAergic input as a result of the low chloride concentration (Chamberlin and Dingledine, 1988).

Epileptiform activity also occurs in low extracellular calcium (0.2mM). This is thought to be a result of nonsynaptic excitatory interactions generating a strong positive feedback in the neuronal network as a result of lost calcium dependent inhibitory and stabilizing mechanisms. There may also be an increased potassium concentration as a result of reduced calcium activated potassium conductances (Konnerth et al.,1986).

Increases in the bicarbonate concentration induce spontaneous activity possibly as a consequence of increasing the pH (Church and McLennan, 1988), although there may be a specific action of the bicarbonate ions on the membrane excitability (Aram and Lodge, 1987).

Finally, reduction in the extracellular magnesium induces spontaneous activity (Stanton et al., 1987). This may be due to a loss of magnesium block on the NMDA receptor operated channel, activating channels that are normally inactive during synaptic transmission (Coan and Collingridge, 1987). Magnesium also acts as an endogenous calcium channel regulator (Hagiwara and Byerly, 1981), so removal will increase calcium influx into cells and presynaptic terminals and increase the overall excitability. There may also be a reduction in the screening charge on the membrane.

Obviously, the in vitro brain slice preparation is very susceptible to changes in the ionic balance. This leads to the possibility that changes in the in vivo CSF ion composition may be a contributing factor to pathological seizures. Extracellular potassium is known to rise during spontaneous discharges (Fisher et al., 1976), although whether this is a cause or effect is not known. Calcium deficiency syndromes are frequently associated with seizures which can be corrected by giving calcium (Millichap, 1969). Eclampsia, seizures occurring in pregnant women, can be treated by giving magnesium salts (Valenzuela and Munson, 1987).

The influence of endogenous ions is not only confined to the major ions. Trace elements, such as copper, zinc, manganese, cobalt and iron are found endogenously and have been implicated in neuronal disorders. Intracranial injections of metal ions, such as iron, copper and cobalt, cause spontaneous seizures (Ward, 1972; Zhao et al., 1985), while clinical symptoms of metal poisoning typically include cerebral seizures (Le Quesne, 1981). A number of studies have looked at the concentrations of endogenous metal ions in epileptic animals and humans; a common feature being an increase in aluminium and zinc, and a decrease in the levels of manganese and cobalt in epileptics (Davidson and Ward, 1988; Dupont and Tanaka, 1985; Hurley et al., 1963). Chung and Johnson (1983) measured the concentration of copper and zinc in the brains of audiogenic and normal mice. They found a small but significantly greater concentration of zinc and copper in the seizure prone mice as compared to the normal mice. The increase was apparent in the whole brain, but the difference was accentuated in the hippocampus and the superior and inferior colliculi. Experiments where two successive generations of normal mice were kept on a high zinc diet failed to cause an increase in brain zinc levels, indicating that the raised metal ion concentration in the audiogenic mice is an inherent rather than an environmental defect. Metal ion concentrations were very constant, never varying more than 2% among animals of the same litter, regardless of brain size variations. The method by which elevated metal ion concentrations might cause epileptic



seizures is not clear. Chung and Johnson (1983) suggested that the involvement of metal ions in enzymatic processes is a likely site for an imbalance of the biochemical homeostasis. In particular, the enzymes glutamine synthetase and glutamate decarboxylase are known to be dependent on the metal ion concentration. An excess of metal ions may conceivably result in a decrease in activity of these enzymes as has been found in biochemical studies, although with concentrations of metal ions 20 times higher than that found in the brain. The result of decreased enzyme activity would be an increase in glutamate and a decrease in GABA concentrations, a situation that would inevitably increase neuronal excitability.

Zinc was found to be the second most concentrated trace element, after iron, in the study by Chung and Johnson (1983), and is also known to be highly concentrated in the synaptic terminals (Haug, 1967; Perez-Clausell and Danscher, 1985). Peters et al. (1987) studied the effect of zinc, applied by pressure ejection, on membrane potential responses to excitatory amino acids in cultured mouse cortical cells. They found that zinc selectively blocked NMDA receptor mediated depolarizations while having no effect on the membrane potential or responses to kainate or quisqualate. This response was very similar to the block imposed by magnesium ions, but zinc was able to further depress the response to NMDA in the presence of magnesium (1mM). This suggests that zinc may be adding to the effect of magnesium at the channel, or possibly acting at the

receptor to reduce NMDA binding. As zinc has been shown to be released from synaptic terminals, both spontaneously (Perez-Clausell and Danscher, 1986), and in response to depolarization (Sloviter, 1985; Assaf and Chung, 1985), the possibility arises that zinc may play a role in the regulation of NMDA responses together with the more ubiquitous magnesium. Unfortunately, this hypothesis is in contrast to the role of zinc and other metal ions as causative agents in epilepsy (Chung and Johnson, 1983), as a block of NMDA receptors would be expected to reduce neuronal excitability. However, it is apparent from the above studies that endogenous levels of metal ions, including the trace elements, have a role to play in the regulation of neuronal excitability, and that changes in these ions can give rise to neuronal abnormalities.

Ligand binding studies have frequently highlighted the importance of endogenous ions for efficient binding of ligands to their receptors. Baudry and Lynch (1979) showed that the binding of glutamate to its receptor is regulated by a number of cations; sodium, lithium, rubidium, caesium and potassium reducing binding with increasing concentrations; calcium and manganese enhancing it. Pretreatment of the membranes with EDTA, to remove membrane bound ions, markedly reduced ligand binding, indicating that endogenously bound ions are important for ligand binding. Similarly, binding to  $\alpha$ -adrenergic receptors has been shown to be enhanced by magnesium and reduced by sodium in

platelets (Tsai and Lefkoistz, 1978) and rat and bovine brain (U, Prichard and Snyder, 1980). Other receptor types reported to be affected by endogenous ions include opiate receptors (Childers and Snyder, 1980), histamine receptors (Chang and Snyder, 1980) and GABA receptors (Hill and Bowery, 1981).

#### 1.1.1. The NMDA receptor operated channel.

The relationship between adenosine, calcium channels and magnesium inevitably involves interactions with other receptor operated membrane channels. It is therefore necessary to provide a brief explanation of the involvement of the most relevant of these, the NMDA receptor operated channel.

The dicarboxylic amino acids, L-glutamate and L-aspartate are widely thought to act as neurotransmitters in the mammalian CNS. At present, there are three subclasses of receptors at which the excitatory amino acids can act; the kainate, quisqualate and NMDA (N-methyl-D-aspartate) receptors, named after the original exogenous ligands used to characterize them. The NMDA receptor is the most well characterized, mainly due to the availability of specific antagonists and its unique property of being sensitive to block by physiological concentrations of magnesium. Studies of the nature of the receptors have shown the NMDA receptor operated channel to be a voltage sensitive ion channel, most probably a calcium channel as it can be blocked by verapamil and cobalt (Dingledine, 1983). The voltage dependent nature

of the NMDA channel differentiates it from the kainate and quisqualate receptor operated channels which give an increase in membrane conductance with subsequent increased neuronal excitability upon addition of the agonists (McDonald et al., 1982). The NMDA channels require a higher level of membrane depolarization before NMDA and other specific agonists can induce an increase in membrane conductance (Dingledine, 1983). Evans et al. (1977) originally reported the ability of magnesium ions to block responses mediated by activation of NMDA receptors, but it was a while before it was realized that the voltage sensitivity of NMDA conductance changes was a result of the voltage dependence of the ability of magnesium to interfere with the channels involved (Nowak et al., 1984; Mayer et al., 1984). This occurred at physiological concentrations of magnesium (1-2mM) and removal of the magnesium allowed NMDA to produce a voltage independent increase in membrane conductance (Crunelli and Mayer, 1984). The distribution of NMDA receptors is particularly high in the hippocampus (Monaghan and Cotman, 1985) and it is thought that the excitatory amino acids are the principal neurotransmitters in the hippocampus, particularly in the CA1 region (Fagg et al., 1986). Synaptic transmission via the NMDA receptor is not thought to be active in "normal" conditions due to the block by magnesium (Koerner and Cotman, 1982; Collingridge et al., 1983). Only in conditions where the necessary depolarization is provided, such as a reduction in synaptic inhibition, high frequency stimulation or a reduction in

uptake, are the NMDA receptors activated (Coan and Collingridge, 1987).

The excitatory nature of glutaminergic and aspartergic neurotransmission has led to the suggestion that it is involved in epileptic seizures (Meldrum, 1984). Kainic acid is a particularly potent excitatory neurotoxin. Systemic administration causes seizures that are associated with neurodegeneration of areas of the limbic system, particularly the hippocampus (Nadler et al., 1978; Lothman et al., 1981), while addition of kainic acid to hippocampal slices causes epileptiform activity (Westbrook and Lothman, 1983). The NMDA receptor, in particular, has been implicated as a mediator of epileptiform activity. The removal of magnesium from solutions bathing in vitro brain slice preparations, in particular neocortical (O'Shaughnessy et al., 1988) and hippocampal slices (Mody et al., 1987), results in spontaneous epileptiform burst activity, as well as multiple potentials following an evoked depolarization (Herron et al., 1985; Schneiderman and MacDonald, 1987). The fact that 2-AP5 and other NMDA antagonists can block these bursts and reduce the secondary evoked potentials suggests strongly that NMDA receptor activation is the main cause of the increased excitability. The increased excitability observed in the absence of GABA inhibition is also thought to be mediated by NMDA receptors. Dingledine et al. (1986) showed that epileptiform burst firing in the absence of GABAergic inhibition appears to be mediated via NMDA

receptors. They studied orthodromically evoked CA1 population potentials in the presence of picrotoxin and bicuculline to abolish GABAergic synaptic inhibition. Electrically evoked, and occasionally spontaneous, epileptiform burst firing occurred that could readily be abolished by 2-AP5, a specific NMDA receptor antagonist. 2-AP5 had no effect on normal disinhibited field potentials. Intracellular recordings of the pyramidal cells indicated a voltage dependent component of the excitatory postsynaptic potential; 2-AP5 selectively abolished this voltage dependent component in disinhibited slices. This indicates that the excitatory transmission in the absence of GABA inhibitory postsynaptic potentials is mediated via NMDA receptors activated by the reduced membrane voltage potential. Ashwood and Wheal (1987) looked at specific lesions of the hippocampal CA3-CA4 region, induced by chronic administration of kainic acid, which causes epileptiform activity, thought to be the result of a loss of GABAergic inhibition. This increased excitability is also thought to be mediated via NMDA receptors.

The strong evidence for a role of NMDA receptors in epileptiform activity has led to the idea of using NMDA receptor antagonists for the control, or possible prevention, of epileptic seizures (Croucher et al., 1982; Meldrum, 1984). Intracellular injections of specific NMDA antagonists, in particular 2-AP5 and 2-AP7 (2-amino-7-phosphonoheptanoic acid), potently blocked

seizures in audiogenic seizure susceptible mice. Their potency was as good as the benzodiazepines, the most potent established anticonvulsants. 2-AP7 was also effective against seizures in photosensitive epileptic baboons when administered i.v., although the potency was much lower due to poor entry into the brain.

Another drug with potent anticonvulsant properties is MK801 (Clineschmidt et al., 1982). This also appears to act on the NMDA receptor operated channel but via a different mechanism to 2-AP5. Mk801 binds to the phencyclidine (PCP) receptor which is thought to be located within the NMDA receptor operated channel (Fagg, 1987) and blocks the channel directly (Heuttner and Bean, 1988). Recent evidence suggests that rapid association with the PCP receptor only occurs through the activated open channel (Javitt and Zukin, 1989). Also, addition of 2-AP5 is thought to "freeze" the channel, holding MK801, and magnesium ions, in the channel (Sokolovsky and Kloog, 1988). Thus it appears that increased NMDA receptor activation is implemented in acute epileptiform activity in in vitro models, and the addition of NMDA specific antagonists can prevent chronic epileptic seizures in susceptible animals.

The question is, what do NMDA receptors do in the CNS, apart from cause epileptic seizures if over activated?. At present it is thought that NMDA receptors are involved in long term potentiation (LTP), whereby tetanic stimuli can potentiate synaptic transmission in a particular pathway for

long periods of time, up to 3 days (Bliss and Gardner-Medwin, 1973). This is a prominent feature of excitatory synapses in the hippocampus and is hypothesized to be involved in memory and learning (Collingridge and Bliss, 1987). LTP is particularly sensitive to NMDA antagonists, which prevent it, while blockade of GABAergic inhibition facilitates LTP (Wigstrom and Gestaffson, 1985). Both effects suggest strongly that NMDA receptors are involved in LTP and this may be a major role of the NMDA receptor, at least in the hippocampus. Adenosine has also been shown to block LTP (Dolphin, 1983), possibly suggesting an association between NMDA and adenosine receptors.

The idea of looking at a possible relationship between adenosine and magnesium was prompted by a comparison of the data from two similar studies looking at the effect of adenosine on epileptiform bursts. Ault and Wang (1986) looked at the effect of adenosine and a range of adenosine analogues on epileptiform bursts induced by bicuculline in the CA3 region of the rat hippocampal slice. They observed a potent inhibition of the bursts; adenosine giving an  $IC_{50}$  of  $1.5\mu M$ ; 2-chloroadenosine,  $0.144\mu M$  and R-phenylisopropyladenosine,  $0.012\mu M$ . O'Shaughnessy et al. (1988) looked at bursts induced by magnesium free ACSF in the rat cerebral cortex slices. They recorded  $IC_{50}$  values for the decrease in burst frequency of  $30\mu M$  for adenosine; 2-chloroadenosine,  $5\mu M$  and R-phenylisopropyladenosine,  $0.24\mu M$ . This represented a 20 fold difference in potency



between the two studies. This could have been due to a number of factors, including the different brain regions used, the different recording techniques, electrodes as opposed to the grease gap technique of recording changes in potential difference, or a difference in the method of inducing epileptiform bursts. The latter possibility, in conjunction with ligand binding studies, was the basis for this study into a possible relationship between adenosine and magnesium.

## 1.2. MATERIALS AND METHODS

### 1.2.1. Chemicals and Biochemicals.

General laboratory reagents were "Analar" grade and were purchased from BDH Chemicals Ltd., UK, unless otherwise stated. Adenosine analogues and other neuroactive compounds were purchased from the Sigma Chemical Co, UK, or Research Biochemicals Inc. unless otherwise stated.

Compounds were dissolved in the appropriate vehicle as a stock solution. In most cases the vehicle was ACSF. When other vehicles were used, control experiments were carried out to ensure there was no effect from the vehicle.

### 1.2.2. Animals.

Rats (male, Wistar 150-250g) were obtained from the breeding colony at St George's Hospital Medical School. These were housed in a controlled environment with a 12 hour light/dark cycle and free access to food and water.

### 1.2.3. Media.

#### Artificial Cerebrospinal Fluid-bicarbonate solution (ACSF).

$\text{KH}_2\text{PO}_4$ , 2.2mM;  $\text{MgSO}_4$ , 1.2mM;  $\text{KCl}$ , 2.0mM; Glucose, 10.0mM;  
 $\text{NaHCO}_3$ , 25.0mM;  $\text{NaCl}$ , 115.0mM;  $\text{CaCl}_2$ , 2.5mM.

#### Nominally magnesium free ACSF-bicarbonate solution.

$\text{KH}_2\text{PO}_4$ , 2.2mM;  $\text{KCl}$ , 2.0mM; Glucose, 10.0mM;  $\text{NaHCO}_3$ , 25.0mM;  
 $\text{NaCl}$ , 115.0mM;  $\text{CaCl}_2$ , 2.5mM.

Contamination by magnesium from other constituents  
calculated from manufacturers data to be 11 $\mu\text{M}$ .

When 8.5mM potassium was used, the  $\text{KCl}$  was adjusted to  
obtain the required concentration.

The above media were saturated with a mixture of 95%  
oxygen/5% carbon dioxide obtained from BOC., Ltd.

### 1.2.4. Recording electrodes.

The recording electrodes were made from fibre containing  
glass capillary tubing, 2mm diam. (Clark Electromedical  
Instruments. U.K.) pulled on a Kopf vertical electrode  
puller. The tip was bumped back under a microscope to a  
diameter of around 2 $\mu\text{m}$ , resistance 1-2 M $\Omega$ . The electrode was  
backfilled with 3M sodium chloride.

#### 1.2.5. Preparation of rat hippocampal brain slices.

Male Wistar rats were decapitated and the brain quickly removed into a petri-dish containing ice cold artificial cerebrospinal fluid (ACSF) saturated with 95% O<sub>2</sub>/5% CO<sub>2</sub>.

The cerebellum was separated by means of a coronal cut at the rostral end of the cerebellum. Next, a sagittal cut was made to separate the two hemispheres. The cortex of each hemisphere was reflected by means of two fine spatulas, and the hippocampus scooped out.

The hippocampus was then transferred to a McIlwain tissue chopper and placed on filter paper. Transverse slices were then prepared (500µm thickness) and the filter paper supporting the serial sectioned hippocampus was transferred into another petri-dish containing sufficient ACSF to just cover the slices. Individual slices were then separated using a pair of fine glass seekers. Only slices from the dorsal region of each hippocampus were used.

Finally, the slices were transferred to an incubation chamber and placed on a filter paper with sufficient ACSF to just cover the slices. They were maintained in an oxygenated, humidified environment at room temperature until required.

This operation from decapitation to incubation took place within five to eight minutes.

#### 1.2.6. Experimental procedure.

After a minimum of 1 hour incubation, single slices were transferred to the recording chamber, volume 1ml, and superfused by gravity feed at 4mls/min with ACSF prewarmed to 30°C. The slices were submerged and held in place with a circle of fine wire that was attached to, and lowered in to place by, a micromanipulator. A concentric bipolar stimulating electrode was placed on the Schaffer collateral/commissural pathway near the CA2 region, and a glass recording microelectrode was placed in the pyramidal cell body layer of the CA1 region (see Fig.1.1 in results).

The orthodromic evoked potentials were displayed, via a Neurolog preamplifier and filters, on a digital storage oscilloscope from which the potentials were recorded on a chart recorder. Stimuli were of 0.06ms duration, delivered at 0.1Hz from a constant current stimulus isolator. Stimulus strength was set to give a submaximal population potential size of about 75% of maximum (150-300pA) to facilitate obtaining reproducible responses to adenosine.

Drugs were bath applied via the gravity feed system and the changes in population potential measured from the chart records.

Control responses to adenosine were obtained before and after the magnesium free periods to ensure no significant changes in the response, e.g. due to a deterioration in slice condition or a shift in electrode position.

Magnesium free solution was superfused for 20 minutes before the first adenosine application in this medium. Test applications of adenosine were for 8 minutes followed by 15 minutes recovery period.

#### 1.2.7. Epileptiform bursts.

Epileptiform bursts were recorded via a single microelectrode positioned in the CA3 region. A satisfactory orthodromic evoked potential was first obtained to confirm the viability of the region, the stimulating electrode was then removed and the nominally magnesium free medium introduced.

After approximately 10 minutes, spontaneous bursts of electrical activity were observed on the oscilloscope. The spontaneous activity was recorded on the chart recorder direct from the neurolog amplifier at x1000. The burst frequency was recorded as deflections of the chart recorder pen. Individual bursts could be stored on the oscilloscope and plotted onto the chart recorder to give a qualitative record of the epileptiform burst.

The number of bursts occurring over a 10 minute period were counted and the burst frequency per minute was determined. Results were presented as a percentage change in the burst frequency with respect to the control period immediately before the drug addition concerned.

#### 1.2.8. Analysis of results.

The storage oscilloscope was set so that only the primary population potential of the evoked field potential was displayed on the screen. This was recorded on the chart recorder as deflections representative of the potential size. The size of the deflection was measured from the baseline to the maximum negative deflection of the population potential. (See fig.1.2 in results.)

Results were analysed as a percentage change in the size of the negative population spike with respect to potential size immediately before each adenosine application.

In some experiments, the signal from the amplifier was passed through an averager and the average of 8 sweeps recorded. A qualitative record of the whole evoked field potential was then plotted out on the chart recorder.

#### 1.2.9. Statistical analysis.

All results are expressed as mean  $\pm$  standard error of the mean (sem) unless stated otherwise. Statistical significance was determined using a paired sample t-test on the Oxstat statistical analysis computer programme.

### 1.3. RESULTS

#### 1.3.1. The evoked CA1 field potential.

The positioning of the stimulating and recording electrodes used to obtain evoked field potentials from the CA1 region is shown in fig. 1.1.

A printout of a typical evoked field potential is shown in fig. 1.2. This was elicited by stimulation of the Schaffer collaterals in the Stratum radiatum. This is a composite potential consisting of a small presynaptic fibre volley followed by the excitatory postsynaptic potential (EPSP) upon which is superimposed the population potential, representative of the depolarization of the pyramidal cell bodies (Andersen et al., 1971).

The portion of the potential recorded and measured on the chart recorder is the distance from the baseline to the maximum deflection of the negative going population potential. The percentage reduction in this is taken to be representative of the level of inhibition at the synapses on the pyramidal dendrites.

The ability of adenosine to reduce the synaptically evoked potential is apparent in fig.1.3. If magnesium is omitted from the ACSF, then the effect of adenosine is markedly reduced.



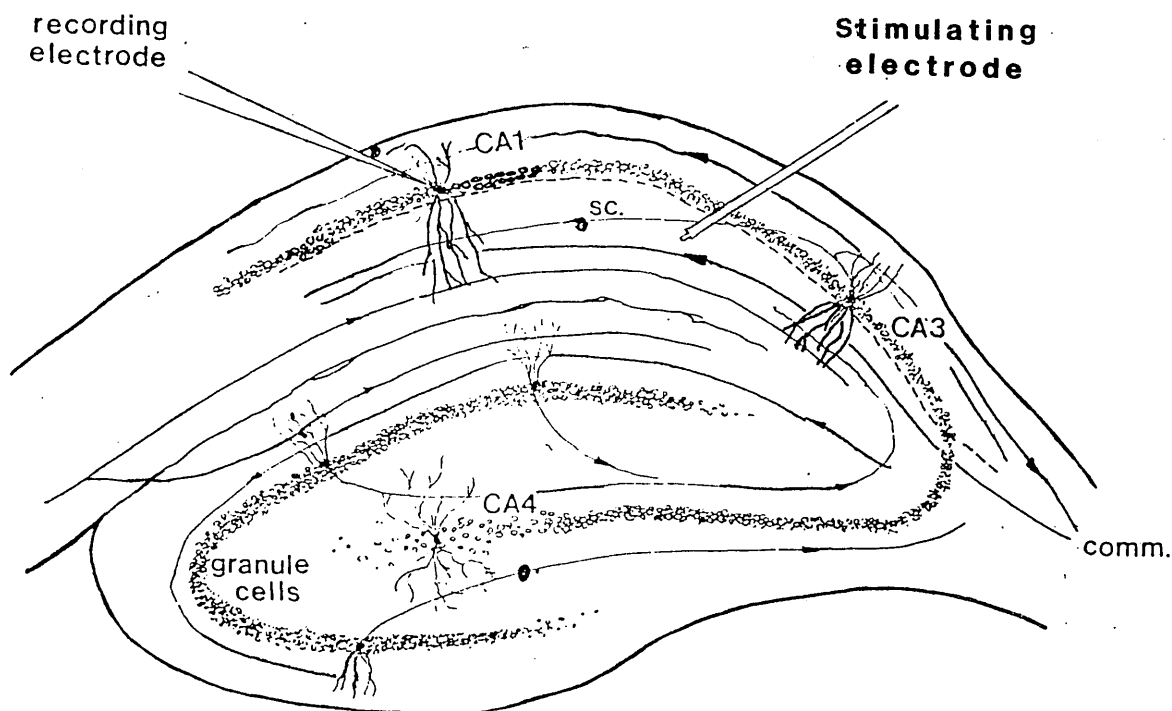


Fig.1.1.

Positioning of electrodes in the hippocampal slice for recording of CA1 evoked field potentials. The Schaffer collaterals (SC) from CA3 pyramidal cells together with commissural fibres (COMM) from the contralateral hippocampus are stimulated and activate pyramidal cells of the CA1 via synaptic connections on the pyramidal cell dendrites. A representative pyramidal cell is shown at the tip of the recording electrode.

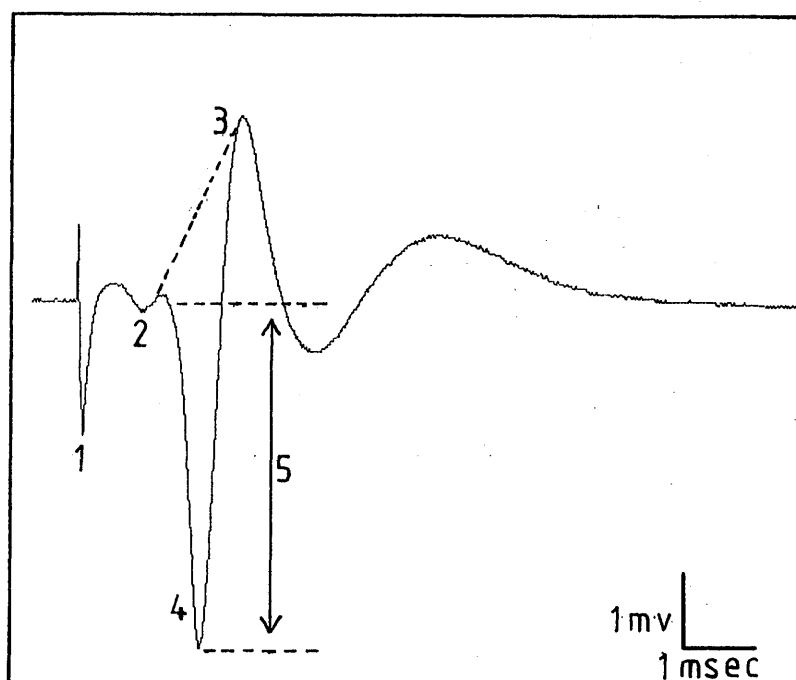


Fig.1.2. Evoked CA1 field potential.

1. Stimulus artifact.
2. Presynaptic fibre volley.
3. Excitatory postsynaptic potential.
4. Population potential.
5. Deflection measured to determine percentage change in potential size.

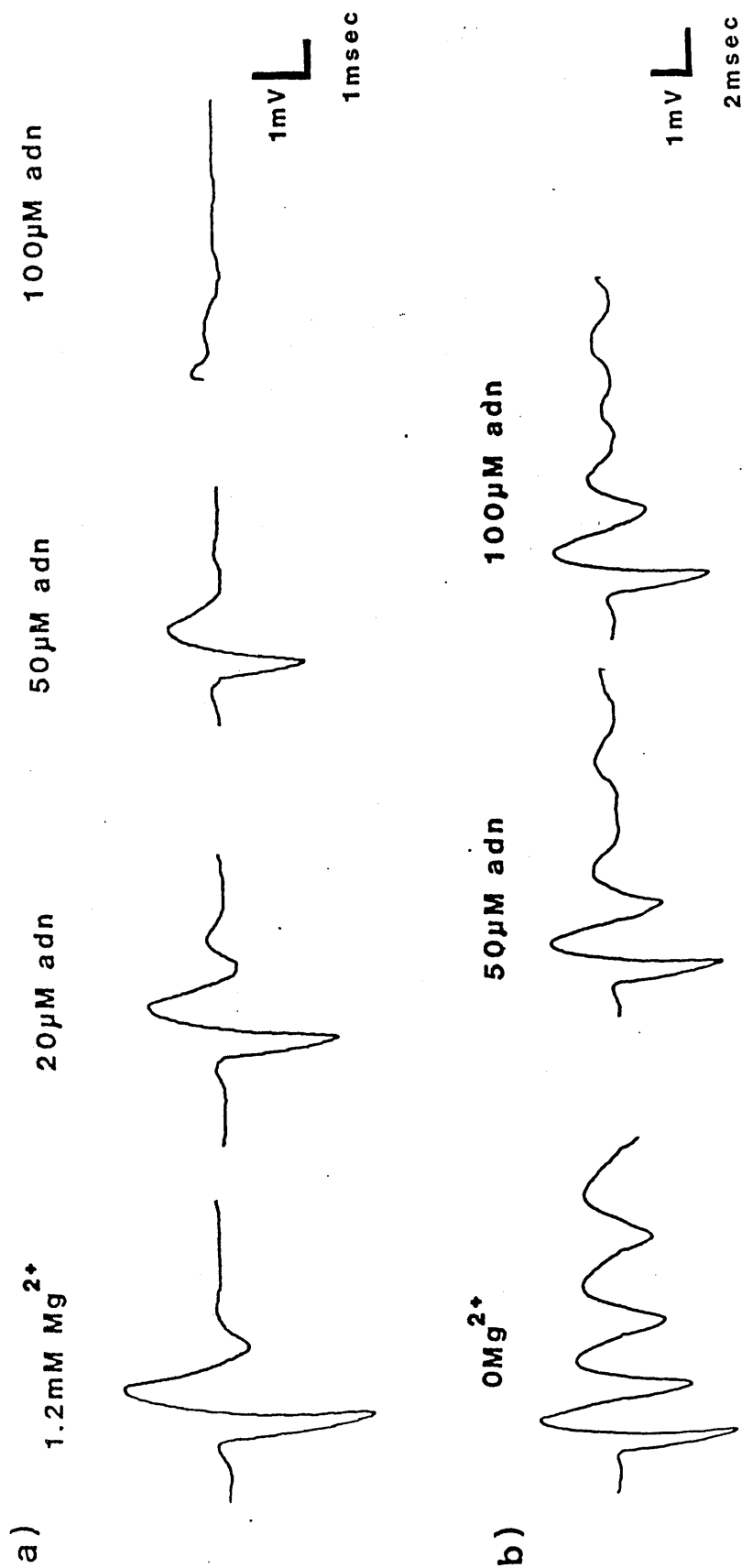


Fig 1.3. Sample records of field population potentials in  
a) control (1.2mM) magnesium and b) nominally magnesium free  
ACSF and the effect of increasing concentrations of  
adenosine. Each trace is an average of 8 evoked responses.  
Stimulus artifacts have been omitted for clarity.

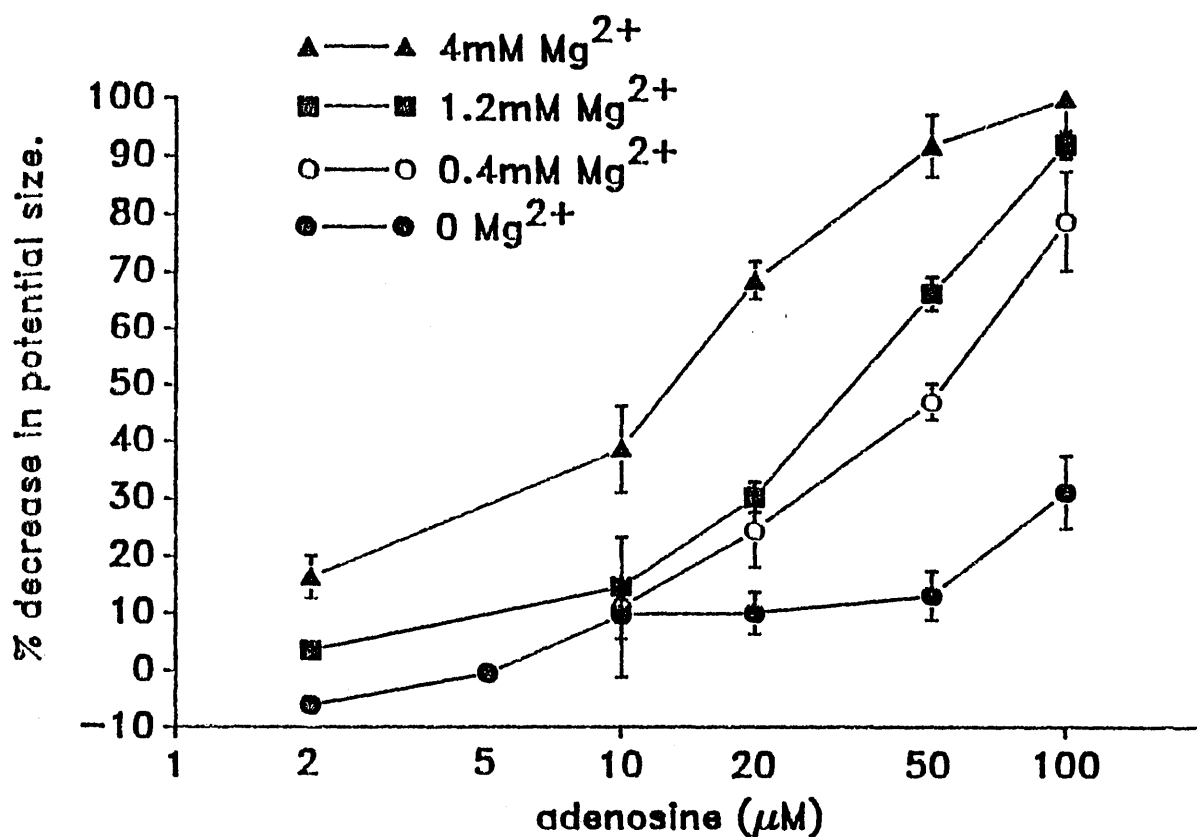
1.3.2. The effect of magnesium on the inhibition of the CA1 population potential by adenosine and 2-chloroadenosine.

Dose response curves to adenosine were determined in the presence of different concentrations of extracellular magnesium (Fig.1.4.).

Control responses to adenosine were performed before and after the period of altered magnesium. Results were discarded if the post-altered magnesium responses were more than 10% different from the control. Adenosine was perfused for 8 minutes per concentration with a washout time of 15 minutes between each concentration. Responses to adenosine were apparent as soon as the adenosine reached the slice and a maximum response was reached within 2-3 minutes. ACSF with altered magnesium concentrations were perfused for 30 minutes before the first application of adenosine. Any changes in the potential size due to changes in magnesium were normally observed within 10-15 minutes.

The inhibition of the population potential by adenosine was enhanced in high magnesium (4mM) and reduced in low magnesium (0.4mM). Omission of magnesium altogether severely attenuated the action of adenosine.

Fig.1.4. Dose response curves of the effect of adenosine on CA1 population potentials at different concentrations of extracellular magnesium.



The effect of adenosine on the evoked CA1 population potential at different concentrations of extracellular magnesium. In control 1.2mM  $\text{Mg}^{2+}$  ACSF, the  $\text{IC}_{50}$  for adenosine is  $37 \pm 3.4 \mu\text{M}$ ,  $n=30$  (mean  $\pm$  s.e.m). In 0.4mM  $\text{Mg}^{2+}$  the  $\text{IC}_{50}$  increases to  $56 \pm 7.5 \mu\text{M}$  ( $n=5$ ). In 4mM  $\text{Mg}^{2+}$  the  $\text{IC}_{50}$  is reduced to  $11.7 \pm 2 \mu\text{M}$  ( $n=5$ ). In magnesium free ACSF inhibition is greatly reduced with 100 $\mu\text{M}$  adenosine giving only a  $31 \pm 6.4\%$  ( $n=10$ ) decrease compared to  $92.1 \pm 2.5\%$  in normal ACSF. Error bars show the s.e.m when these are greater than the symbol size.

There is a significant difference ( $P < 0.05$ , paired t-test) between the normal and 4mM magnesium, and between the normal and magnesium free points, except at 10 $\mu\text{M}$  adenosine.

At concentrations of adenosine lower than 10 $\mu\text{M}$  in magnesium free ACSF, there was a small increase in the potential size.

The slope of the magnesium free dose response curve appeared to be reduced with respect to the control curve. To determine whether this change was statistically significant, the points between 20 $\mu$ M and 100 $\mu$ M were plotted for each magnesium free experiment. The slope of each line was determined for control ( $0.78 \pm 0.05$ ,  $n=10$ ) and magnesium free ( $0.25 \pm 0.07$ ,  $n=10$ ) experiments and compared using a paired t-test.

The regression coefficients for the majority of the lines were close to 1, so it was valid to fit a straight line to the points.

There was a highly significant difference ( $P<0.001$ ) between the slopes with the magnesium free dose response curve being significantly shallower than the control.

A Schild plot of the data was not possible as there were insufficient concentrations of magnesium (the "antagonist") below the control 1.2mM magnesium to give a suitable plot.

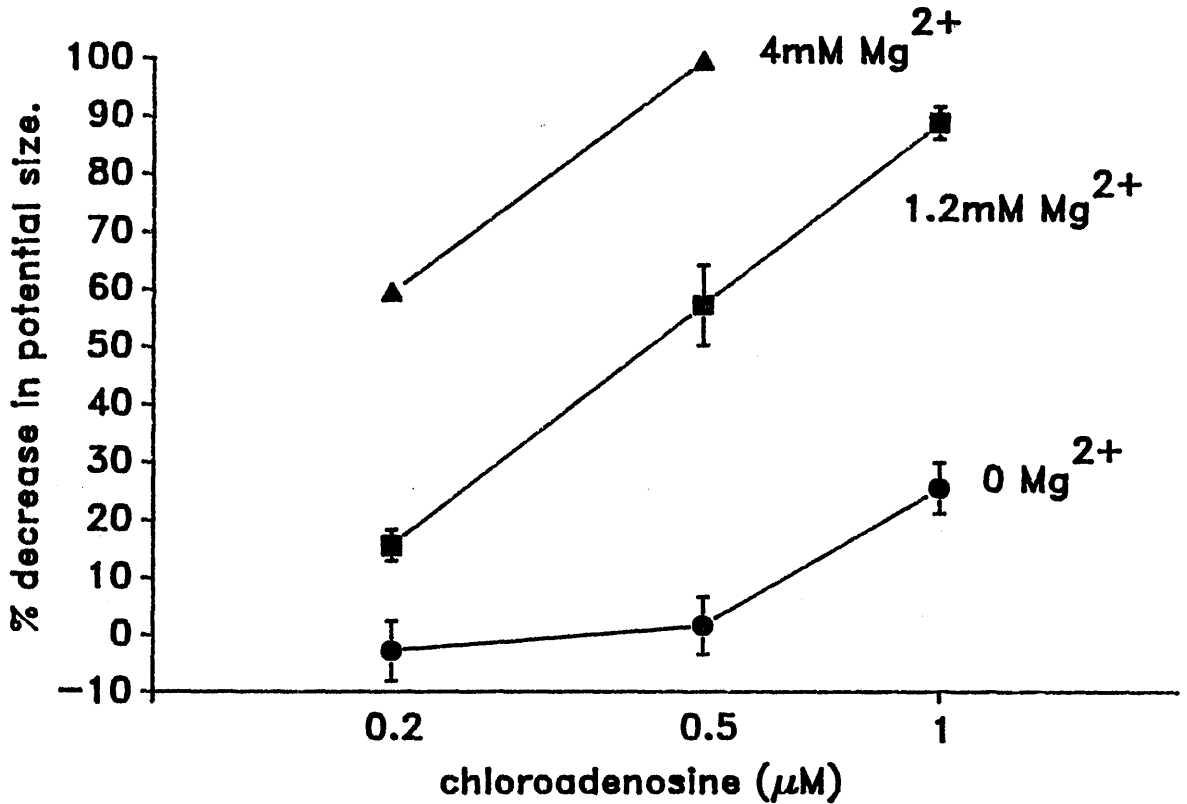
Adenosine is rapidly taken up and metabolized in vitro (Bender et al., 1981). The possibility that uptake processes were being enhanced by the low magnesium was controlled for by using 2-chloroadenosine, an analogue of adenosine with a low affinity for the uptake system (Jarvis et al., 1985).

The potency of 2-chloroadenosine was also affected by varying the magnesium concentration (Fig.1.5.).

Further experiments were carried out to determine the maximum attainable depression by adenosine in magnesium free ACSF (Fig.1.6.). Adenosine was added at increasing concentrations, with washout periods of 15 minutes between each concentration, up to a concentration of 1mM or until there was a 100% inhibition of the potential.

The  $IC_{50}$  for adenosine in magnesium free ACSF was  $125 \pm 15\mu M$  (n=6). In some slices a complete inhibition of the population potential was not possible even at concentrations of 1mM adenosine.

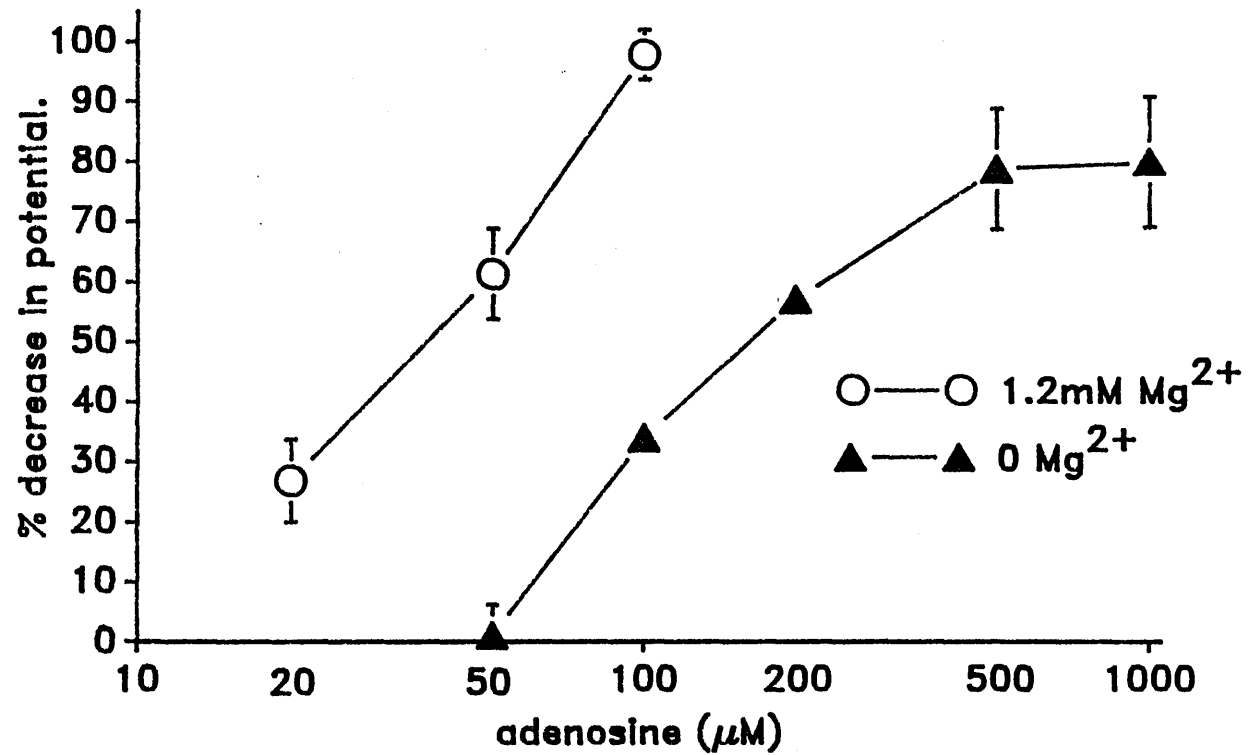
Fig.1.5. Dose response curve for the effect of 2-chloroadenosine on the population potential at different magnesium concentrations.



The effect of 2-chloroadenosine on the CA1 evoked population potential in different extracellular magnesium concentrations. The  $\text{IC}_{50}$  for 2-chloroadenosine in normal magnesium is  $320 \pm 50\text{nM}$ . Points are mean  $\pm$  s.e.m of 5 slices. Error bars show the s.e.m when these are greater than the symbol size. All points are significantly different from control (1.2mM) magnesium levels. ( $P < 0.05$  PTT).



Fig.1.6. The effect of a lack of magnesium on the maximum attainable inhibition of the CA1 population potential by adenosine.



The percentage inhibition of the CA1 population potential by increasing concentrations of adenosine. The  $\text{IC}_{50}$  for adenosine is  $35 \pm 4.6 \mu\text{M}$  in 1.2mM Magnesium and  $125 \pm 15 \mu\text{M}$  in magnesium free ACSF. Points are mean  $\pm$  sem of 6 determinations.

### 1.3.3. The contribution of increased cell excitability to the reduction in adenosine inhibition.

The effect of altering the magnesium concentration is to increase or decrease the size of the population potential. Altering the magnesium concentration from 1.2mM to nominally magnesium free resulted in an increase in potential size of  $11.8 \pm 2.2\%$  (n=14) ( $P < 0.05$ , paired t-test) together with the appearance of secondary potentials (Fig.1.3.). An increase in magnesium from 1.2mM to 4mM decreased the potential by  $19.2 \pm 5.16\%$  (n=7) ( $P < 0.05$ , paired t-test).

The possibility arises that the apparent efficacy of adenosine in magnesium free ACSF is being reduced by the overall increase in excitability. To control for this, a number of experiments were carried out to reduce the excitability without reintroducing the magnesium.

### 1.3.3 (i). NMDA receptor mediated excitation.

One possible result of removing the extracellular magnesium is to open NMDA receptor operated channels, which would result in increased cell excitability (Stanton et al., 1987). To control for this, 2-aminophosphonopentanoic acid (2-AP5), an antagonist at the NMDA receptor was added to the ACSF at a concentration of 50 $\mu$ M.

% decrease by adenosine (50 $\mu$ M)			% decrease by 2-AP5 in Mg free
control(1.2mM Mg)	Mg free	Mg Free + 2-AP5(50 $\mu$ M)	
81.4 $\pm$ 6.47	13.2 $\pm$ 6.8***	14.5 $\pm$ 4.5***	0.12 $\pm$ 2.1

Table 1.1. Values are the percentage decrease of the population potential. Mean  $\pm$  sem of 6 determinations. Values are significantly different from control. (\*\*\*) =  $P < 0.001$ , PTT).

In this and subsequent tables, "control" refers to the normal response to adenosine in the standard ACSF. The size of the evoked population potential in the absence of any compounds is taken to be a 0% change in potential size.

The addition of 2-AP5 had no effect on the size of the potential, or the effect of adenosine in magnesium free ACSF.

### 1.3.3.(ii). Reduced extracellular calcium.

A second approach was to reduce the calcium content of the ACSF to reduce the calcium influx through presynaptic calcium channels and subsequently reduce neurotransmitter release. The calcium concentration of the magnesium free ACSF was reduced until the primary population potential was reduced to a size that was close to that observed in normal ACSF. A calcium concentration of 1.2mM was normally sufficient to reduce the potential.

% decrease by adenosine (50 $\mu$ M)			% decrease by Mg free/low Ca
control(1.2mM Mg)	Mg free	Mg Free/ low(1.2mM)Ca	
84.6 $\pm$ 1.86	26.9 $\pm$ 7.4***	28.8 $\pm$ 3.4***	31.4 $\pm$ 5.9

Table 1.2. Values are the percentage decrease of the population potential. Mean  $\pm$  sem of 6 determinations.

(\*\*\* =  $P < 0.01$ , PTT).

The reduced calcium did not return the characteristics of the potential to that seen in normal ACSF, i.e. a single primary potential, but reduced the overall size of both primary and secondary population spikes. The ability of adenosine to reduce the population potential was not restored in low calcium.

### 1.3.3.(iii). Addition of cobalt.

The final method was to add a metal ion known to have calcium channel blocking properties (Tsien et al., 1987). Cobalt was used, as this has a relatively weak action and does not totally abolish the potential. It was also the only ion that could be added to the bicarbonate ACSF without precipitation of the bicarbonate.

% decrease by adenosine (50 $\mu$ M)			% decrease by cobalt(500 $\mu$ M)
control(1.2mM Mg)	Mg free	Mg Free + cobalt (500 $\mu$ M)	
73 $\pm$ 8.5	26.8 $\pm$ 7.8***	82.0 $\pm$ 6.1 <sup>ns</sup>	21.9 $\pm$ 3.7

Table 1.3. Values are the percentage decrease of the population potential. Mean  $\pm$  sem of 6 determinations.

(\*\*\* =  $P < 0.001$  PTT, ns = not significantly different from control).

In this instance, the potency of adenosine was restored to control levels.

From these results it appears that the increase in excitability is not due to an increase in NMDA receptor mediated action and that a reduction in synaptic transmission by lowering the calcium concentration does not restore the potency of adenosine. However, a reduction in excitability by blocking calcium influx with cobalt does return adenosine inhibition to control levels.

#### 1.3.4. The action of adenosine on epileptiform bursts.

The original idea for this study was to determine if there was any difference in the potency of adenosine on epileptiform bursts induced by different methods. The effect of adenosine and 2-chloroadenosine on epileptiform bursts induced by magnesium free ACSF in the CA3 region was determined (Fig.1.7).

The  $IC_{50}$  values for inhibition of the spontaneous activity were  $3.6 \pm 0.22\mu M$  ( $n=4$ ) for adenosine and  $247 \pm 22nM$  ( $n=4$ ) for 2-chloroadenosine.

Epileptiform activity is normally recorded in the CA3 region because bursts are generated here and are stronger and more likely to occur (Wong and Traub, 1983). Epileptiform activity is occasionally apparent in the CA1, possibly triggered by spontaneously depolarizing cells in the CA3. In some slices bursts were apparent in both the CA1 and CA3. Responses to adenosine were determined in both regions in these slices to determine if there was any marked difference in potency (Fig 1.7c).

There was no significant difference in the potency of adenosine or 2-chloroadenosine between the CA1 and CA3 regions.

Fig.1.7. The anticonvulsant action of adenosine and 2-chloroadenosine on magnesium free ACSF induced spontaneous activity in the CA3.

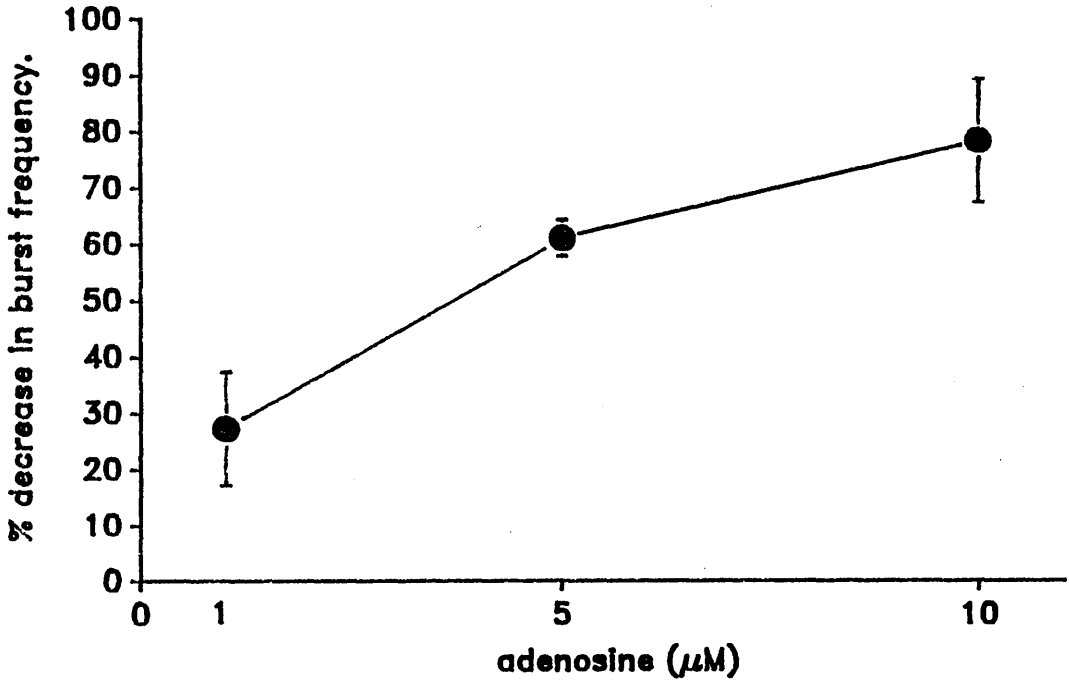


Fig.1.7a. The inhibition of spontaneous activity by adenosine. Values are the percentage decrease of the burst frequency. Points are mean  $\pm$  sem of 5 determinations. The  $\text{IC}_{50}$  is  $3.6 \pm 0.22 \mu\text{M}$  ( $n=5$ ).

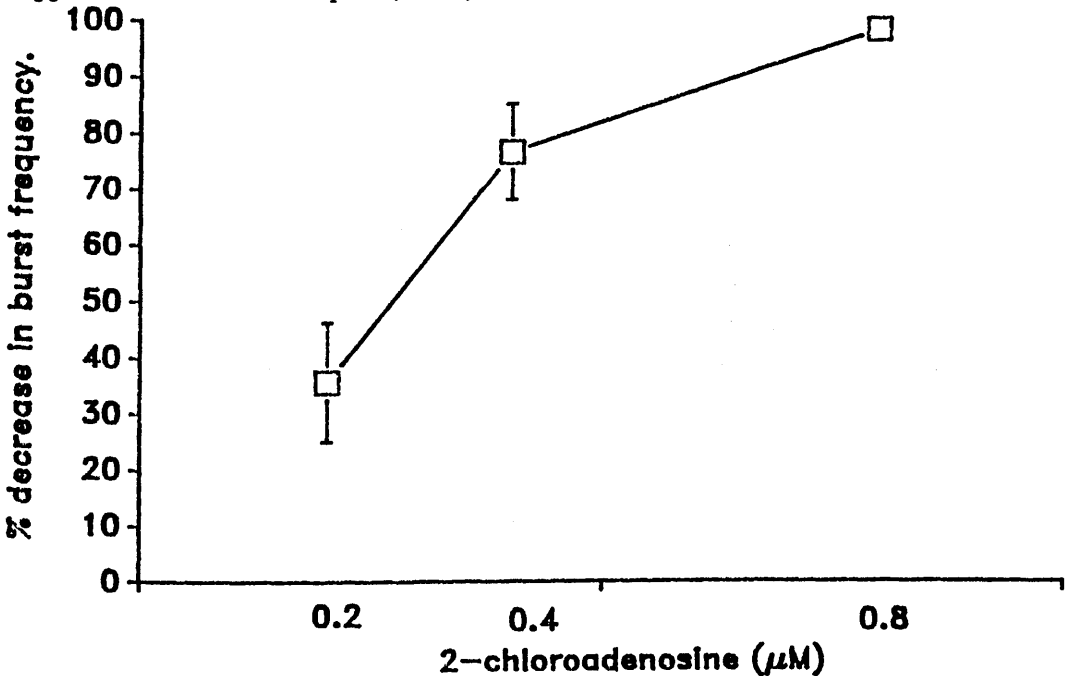


Fig.1.7b. The inhibition of spontaneous activity by 2-chloroadenosine. Values are the percentage decrease of the burst frequency. Points are mean  $\pm$  sem of 5 determinations. The  $\text{IC}_{50}$  is  $247 \pm 22 \text{ nM}$  ( $n=5$ ).

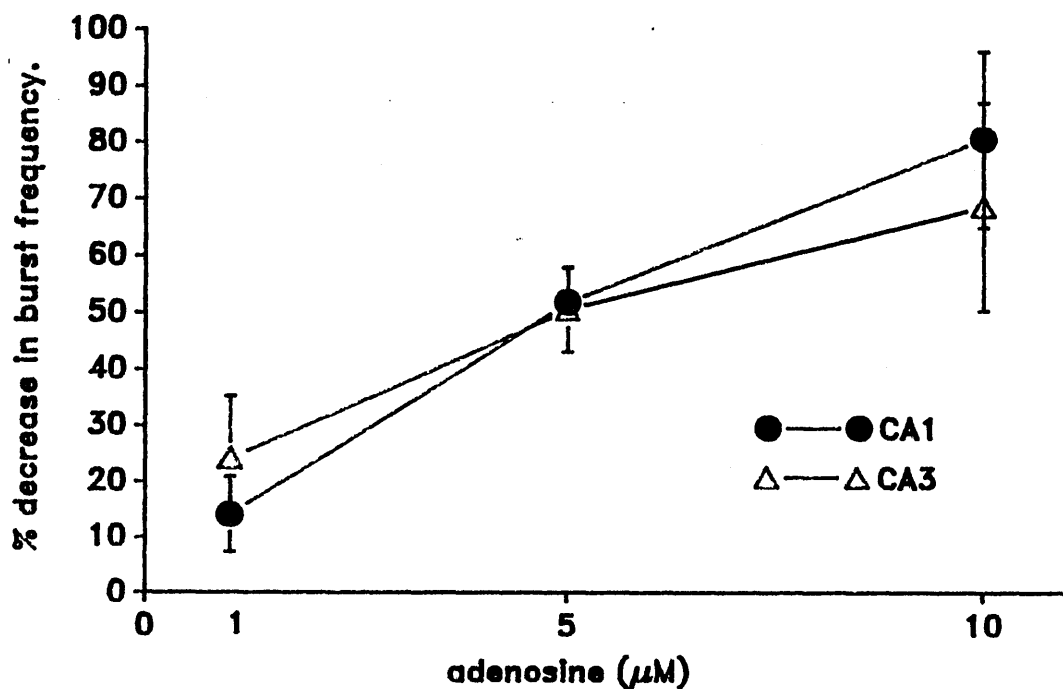


Fig.1.7c. The inhibition of spontaneous activity by adenosine in the CA1 as compared to the CA3. Values are the percentage decrease of burst frequency. Points are mean  $\pm$  sem of 5 determinations.



### 1.3.5. Excitation by adenosine

Returning to the low calcium experiments, an interesting effect of adenosine was observed when it was added to slices bathed in magnesium free/1.2mM calcium ACSF. In a small number of slices, the adenosine produced a marked increase in potential size. It was decided to study this further to determine the reason for this effect.

In 35 of 50 slices studied, addition of adenosine (50 $\mu$ M) induced a response comparable to that seen in magnesium free ACSF, i.e. a small or absent decrease in the potential. However, in the remaining 15 slices, there was an increase in potential size with adenosine or a related analogue, adenosine giving an increase of  $65 \pm 14.2\%$  (n=6). The increase could be blocked by superfusion with 8-phenyltheophylline (10 $\mu$ M) but varying the adenosine concentration did not affect the size of the increase, indicating a lack of dose dependency.

One possibility was that an A<sub>2</sub> receptor response, increasing neuronal excitability, was being observed in the absence of the inhibitory A<sub>1</sub> response. The addition to the superfusing medium of the A<sub>2</sub> receptor agonists 2-phenylaminoadenosine (CV-1808, 10 $\mu$ M), fluorenyl-methyladenosine (PD117,413, 50 $\mu$ M) and N-ethylcarboxamidoadenosine (NECA, 0.1 $\mu$ M), gave variable increases in potential size (NECA  $23 \pm 9\%$ , n=3; CV-1808  $84 \pm 18.7\%$ , n=3; PD117 400%, n=1). Responses were not always repeatable upon addition of a second dose and, in some cases, there was a latency of up to 5 minutes before an increase occurred.

Fig.1.8. shows sample chart records of the increase in potential size seen with some adenosine analogues.

In some slices, the  $A_1$  antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) 10nM, was added to try and enhance any  $A_2$  receptor response. No enhancement of excitation by adenosine was observed and addition of the xanthine did not increase the occurrence of adenosine excitation in control, magnesium free or magnesium free/low calcium conditions, again lending no support to a possible  $A_2$  receptor action.

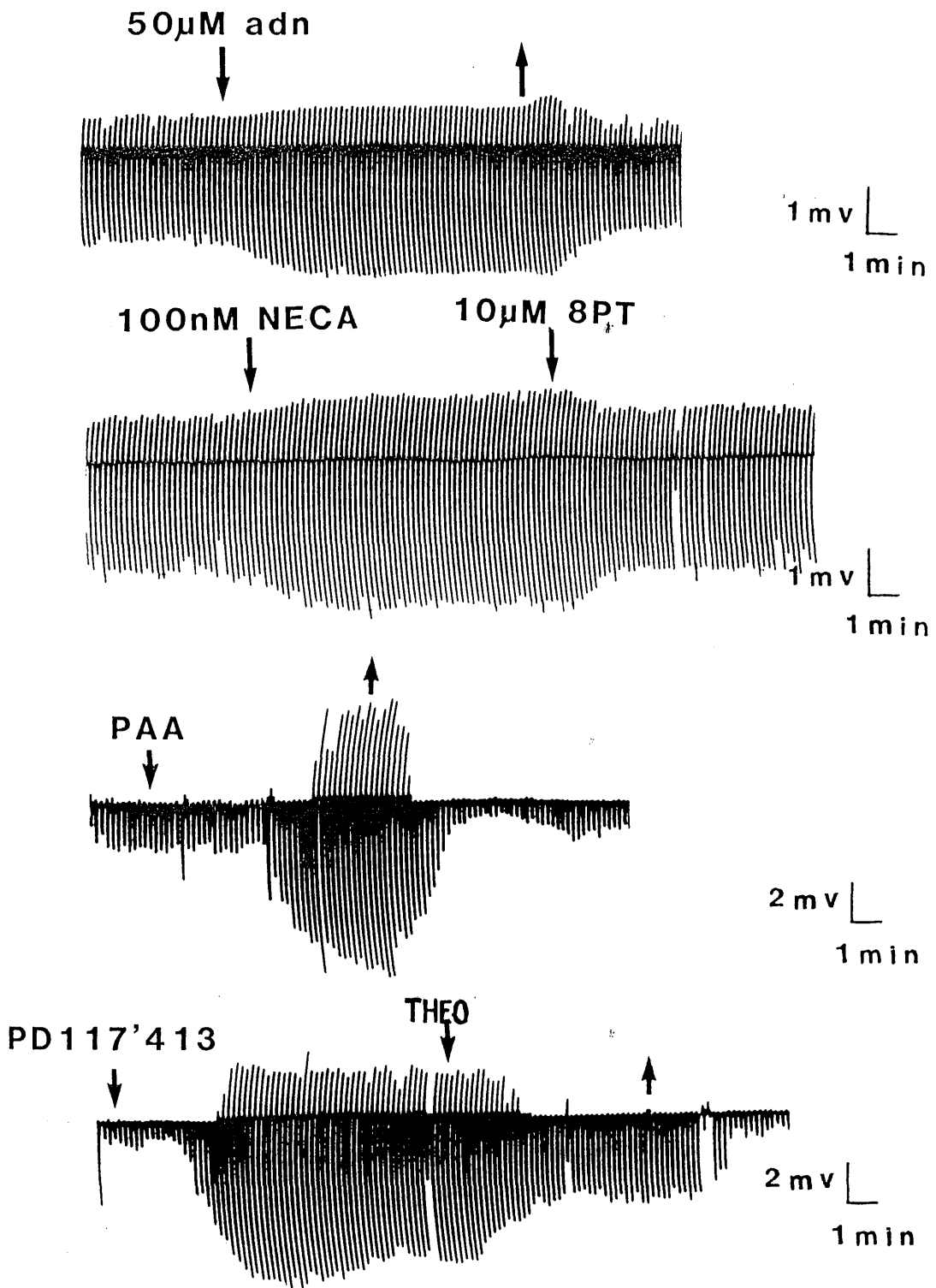


Fig.1.8.

Chart records of responses to adenosine analogues in magnesium free / 1.2mM calcium ACSF.

- a) 50 $\mu$ M adenosine.
- b) 0.1 $\mu$ M NECA, antagonized by 10 $\mu$ M 8-phenyltheophylline.
- c) 10 $\mu$ M 2-phenylaminoadenosine (CV-1808).
- d) 10 $\mu$ M PD117,413, partially antagonized by 50 $\mu$ M theophylline.

The pattern of delayed onset and lack of dose dependency gave the impression of a removal of inhibition rather than an active excitation.

The recording conditions were altered to record activity in the slice direct from the amplifier. This revealed spontaneous depolarisations at a frequency of around 7Hz in the CA1 region during superfusion with magnesium free/low calcium ACSF. These bursts were completely inhibited by 50 $\mu$ M adenosine and the cessation of the bursts corresponded with an increase in the evoked potential size (Fig.1.9.).

The addition of the NMDA receptor antagonist 2-AP5 (50 $\mu$ M), the PCP receptor antagonist MK801 (1 $\mu$ M), the GABA<sub>B</sub> agonist baclofen (10 $\mu$ M), the anaesthetic procaine (500 $\mu$ M), and the endogenous anticonvulsant kynurenic acid (500 $\mu$ M) all inhibited the epileptiform bursts and gave a parallel increase in evoked potential size.

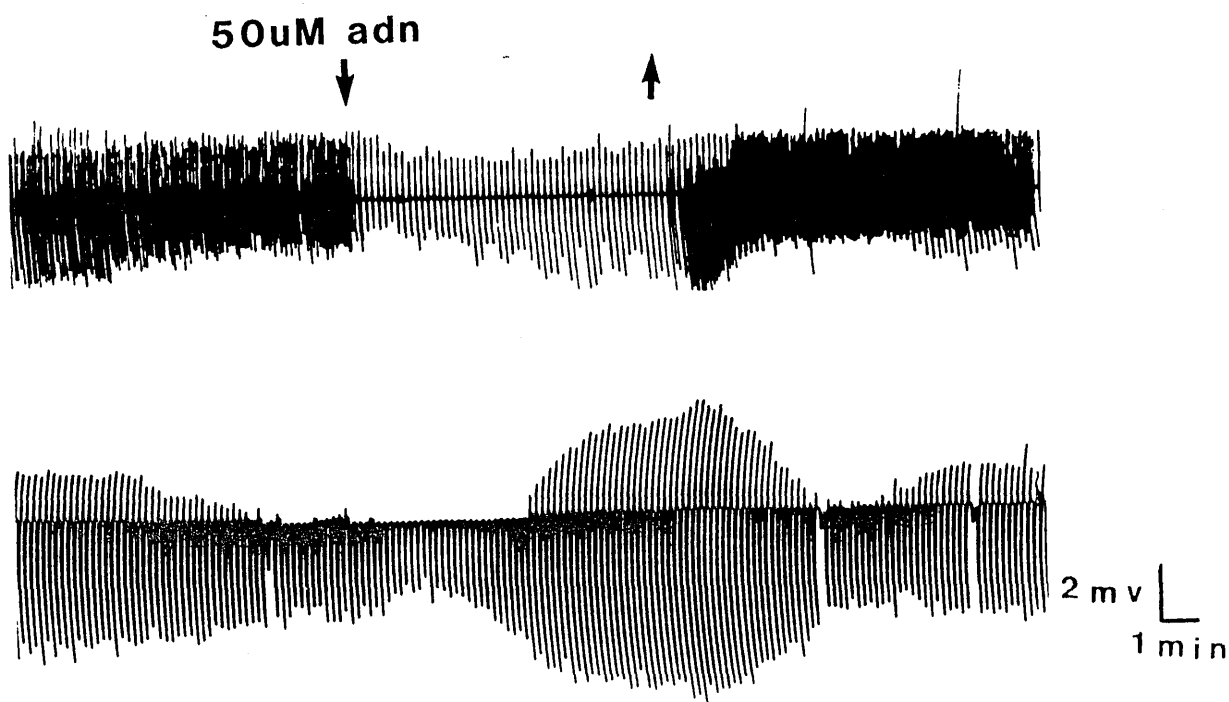


Fig.1.9.

Simultaneous recordings of spontaneous activity (top) and evoked population potentials (bottom). Effect of 50μM adenosine.

Top trace shows activity recorded directly from the amplifier, the majority of deflections represent the evoked potential and stimulus artifact. A high level (0.7Hz) of spontaneous activity is also present that is completely abolished by adenosine.

Bottom trace records the population potential only, recorded via a storage oscilloscope as normal.

#### 1.3.6. Experiments to reduce spontaneous activity without affecting evoked potentials.

The possibility that the spontaneous activity had influenced the results of the earlier magnesium free/low calcium experiments (Table 1.2.) had to be considered. These experiments were repeated under conditions where spontaneous activity was reduced. Two methods were used to inhibit spontaneous activity in magnesium free/low calcium conditions without affecting the recording of evoked potentials.

### 1.3.6.(i)Removal of the CA3 region.

The CA2-CA3 region of the slice has been shown by Wong and Traub, (1983) to be the initiation site for spontaneous activity. This involves a small group of cells that are activated when inhibitory inputs are blocked by bicuculline or penicillin, which can then trigger adjacent cells in the CA1. As spontaneous activity in magnesium free ACSF was more apparent in the CA2-CA3 region, it is reasonable to assume that these cells also initiate the bursts that occur in the absence of magnesium. This region was cut from a number of slices within 10 minutes of the initial preparation, and the slices left to recover for 1 hour. CA1 orthodromic evoked potentials were then recorded as normal.

In magnesium free/low calcium media, spontaneous depolarisations were still apparent, although smaller (0.8mv compared to 2.6mv) and less frequent ( $0.11 \pm 0.001$  Hz,  $n=3$ ) than in intact slices. The bursts were apparent only when the stimulator was turned off, not during evoked potentials. The addition of 50 $\mu$ M adenosine failed to reduce the evoked potential size, while in some slices there was a small increase in potential size. However, as a small level of spontaneous activity was still present, the results from these experiments were not quantitatively analysed.

### 1.3.6.(ii)Baclofen

Baclofen can reduce spontaneous activity at low concentrations, while requiring a much higher concentration before any effect on synaptic transmission is observed (Scott Swartzwelder et al., 1986). 1 $\mu$ M baclofen was added to the bathing medium and inhibited spontaneous depolarisations without significantly reducing the evoked potential. In these experiments, the calcium concentration had to be reduced to 0.8mM before a significant reduction in the potential size was obtained.

% decrease by adenosine (50 $\mu$ M)			% decrease by Mg free/low Ca
control(1.2mM Mg)	Mg free +baclofen	Mg Free/ low(0.8mM)Ca +baclofen	
57.4 $\pm$ 7.45	7.5 $\pm$ 3***	11.8 $\pm$ 3.9***	29.6 $\pm$ 8.2

Table 1.4. Values are the percentage decrease of the population potential. Mean  $\pm$  sem of 6 determinations.

(\*\*\* =  $P < 0.01$  PTT)

Baclofen (1 $\mu$ M) was present throughout the magnesium free period of the experiment and had no effect on the evoked potential in magnesium free ACSF. No spontaneous activity was observed in magnesium free or magnesium free/low calcium ACSF. The inhibition by adenosine was still significantly lower than in magnesium containing ACSF after the potential size had been reduced.

This data supports the earlier low calcium experiments (Table 1.2.) confirming the experimental evidence that the action of adenosine in magnesium free ACSF is not restored when synaptic transmission is reduced by low calcium.



### 1.3.7. Interactions between NMDA channels and adenosine.

Following a report that 2-AP5 can block magnesium free ACSF induced bursts if added prior to the magnesium free ACSF but is much less effective if added afterwards (Stratton et al., 1989), the 2-AP5 experiments were repeated with 2-AP5 being added before or with the introduction of the magnesium free ACSF.

Control 50 $\mu$ M adn	0Mg + 2-AP5(50 $\mu$ M)	0Mg + 2-AP5(50 $\mu$ M) 50 $\mu$ M adn	0Mg 50 $\mu$ M adn
64.6 $\pm$ 4.8	5.4 $\pm$ 2.5	55.8 $\pm$ 6.8**	12.4 $\pm$ 1.9***

Table 1.5. Values are the percentage decrease of the population potential. Mean  $\pm$  sem of 6 determinations.

(\*\*\* =  $P < 0.001$ , \*\* =  $P < 0.05$ , PTT).

There was now no increase in the potential size upon going into magnesium free ACSF when 2-AP5 was present. The addition of adenosine produced an inhibition that was still significantly less than the control, but the inhibition was close to the control level of inhibition and was significantly greater than that observed in magnesium free ACSF when the 2-AP5 was removed ( $P < 0.01$ , PTT).

Thus, it appears from these results that the relationship between adenosine and magnesium is in fact linked to the NMDA receptor operated channel, contrary to the earlier results in table 1.1. The possibility arises that activation of NMDA receptors inactivates or reduces the efficacy of adenosine. To study this possibility, two approaches were used.

1.3.7.(i)The action of adenosine in magnesium free ACSF when NMDA channels are blocked.

To block NMDA receptor operated channels, the phencyclidine (PCP) receptor ligand MK801 was used together with 2-AP5. This combination of NMDA antagonists should remove any activation of NMDA receptor operated channels regardless of the presence of magnesium.

In magnesium free ACSF, MK801 (10 $\mu$ M) was added and a brief 2 minute pulse of NMDA (10 $\mu$ M) was given that partially depressed the potential. This was repeated at 10 minute intervals until the depression by NMDA was abolished, at which point MK801 was deemed to be blocking the majority of NMDA receptor operated channels. 2-AP5 (20 $\mu$ M) was then added and the response to adenosine determined.

% decrease by 50 $\mu$ M adenosine		
Control (1.2mM Mg)	Mg free ACSF	MK801 + 2-AP5 in Mg free ACSF
55.3 $\pm$ 7.1	11.8 $\pm$ 5.2***	41.8 $\pm$ 7.5 <sup>ns</sup>

Table 1.6. Values are the percentage decrease of the population potential. Mean  $\pm$  sem of 6 determinations.

(\*\*\* =  $P < 0.01$  PTT, ns = not significantly different from control)

Antagonism of NMDA receptor and channel activation by 2-AP5 and MK801 in magnesium free ACSF effectively restored the potency of adenosine to control levels. MK801 and 2-AP5, on their own, had no effect on the potential.

1.3.7.(ii) The action of NMDA channel activation on inhibition by adenosine.

The second approach was to activate NMDA receptor operated channels in the presence of normal (1.2mM) magnesium concentrations and to determine the effect on the inhibitory action of adenosine.

NMDA was added at a concentration of 5 $\mu$ M and perfused for 10 minutes until a stable depression of the potential size by NMDA had been obtained. Adenosine was then added in the presence of NMDA.

control 50 $\mu$ M adenosine	5 $\mu$ M NMDA	5 $\mu$ M NMDA+ 50 $\mu$ M adenosine
53.2 $\pm$ 5.26	15.6 $\pm$ 3.54	32 $\pm$ 2.14***

Table 1.7. Values are the percentage decrease of the population potential. Mean  $\pm$  sem of 5 determinations. (\*\*\* =  $P < 0.01$  PTT).

NMDA (5 $\mu$ M) gave a small decrease in the size of the population potential. In the presence of NMDA the effect of 50 $\mu$ M adenosine was significantly reduced in comparison to control.

On a separate set of slices, adenosine (50 $\mu$ M) was perfused to give a steady depression of the population potential for at least 10 minutes. NMDA (5 $\mu$ M) was then perfused in the presence of adenosine.

50 $\mu$ M adenosine	5 $\mu$ M NMDA+ 50 $\mu$ M adenosine
61.0 $\pm$ 5.5	39.0 $\pm$ 5.2***

Table 1.8. Values are the percentage decrease of the population potential. Mean  $\pm$  sem of 5 determinations. (\*\*\*) =  $P < 0.001$  PTT).

The depression by adenosine was attenuated by NMDA within a minute of NMDA onset. This attenuation could be maintained for as long as the NMDA was perfused. Slightly higher concentrations of NMDA (8-10 $\mu$ M) resulted in a total abolition of the population potential.

1.3.8. The effect of high potassium induced excitability on adenosine inhibition with NMDA channels inhibited.

The above results support the idea that activation of NMDA receptor operated channels can reduce the inhibitory action of adenosine. However, it is possible that increased excitability mediated via NMDA channel activation is lowering the action of adenosine by a simple masking action. To control for this, the excitability of the slice was raised by increasing the potassium concentration of the normal ACSF to 8.5mM. NMDA channel action was blocked by adding 2-AP5 prior to the 8.5mM potassium. The action of adenosine was then determined under these conditions.

% decrease by adenosine (50 $\mu$ M)			
control	50 $\mu$ M 2-AP5	8.5mM K <sup>+</sup> +50 $\mu$ M 2-AP5	8.5mM K <sup>+</sup>
61.3 $\pm$ 3.49	60.3 $\pm$ 2.17	51.7 $\pm$ 1.7***	6.0 $\pm$ 2.03***

Table 1.9. Values are the percentage decrease of the population potential. Mean  $\pm$  sem of 4 determinations.

(\*\*\* =  $P < 0.01$  PTT).

Increasing the potassium concentration caused an increase in the excitability of the slice in the presence of 2-AP5 apparent by the appearance of spontaneous activity. There was no significant increase in the evoked potential size. The inhibitory action of adenosine was only slightly reduced in 8.5mM potassium with 2-AP5 present. When the 2-AP5 was removed there was no noticeable change in the potential size or increase in spontaneous activity, but the action of adenosine was significantly attenuated.

#### 1.4. DISCUSSION

The key observation of the experiments reported in this chapter is the marked reduction in the potency of adenosine and 2-chloroadenosine in reducing the hippocampal CA1 evoked population potential when magnesium is omitted from the ACSF. The possible reasons for this, and the possible implications for the role of adenosine as an endogenous neuromodulator, are discussed below.

Omitting the magnesium from the ACSF bathing the hippocampal slice can be expected to increase the overall excitability of the cells. This could be due to three principal effects: 1) A reduction in surface charge. The surface charge on a membrane will determine the level of excitability of the cell. This charge, determined by the charge on the phospholipids and proteins making up the membrane, will be influenced by the ions present in the surrounding medium. A decrease in the number of divalent cations, principally magnesium and calcium, will decrease the stability of the neurone, making it more likely to depolarize (McLaughlin et al., 1971); 2) An increase in calcium influx across voltage sensitive calcium channels, magnesium acting as an endogenous regulator of calcium influx (Hagiwara and Byerly, 1981); or 3) An increase in activity of the NMDA receptor operated calcium channel. These last two possibilities may occur together. Enhanced presynaptic calcium influx across voltage sensitive calcium channels would increase neurotransmitter release while NMDA

receptor channels, thought to be located primarily on the postsynaptic membrane (Fagg and Matus, 1984), would be more readily activated in the absence of magnesium resulting in an increased excitability of the pyramidal cells in response to the increased neurotransmitter release.

Whatever the reason for the increased cell excitability, the effect may simply be to mask the inhibition by adenosine such that, although the adenosine is applying the same level of inhibition at a given concentration, the increased excitability makes the inhibition less apparent. From the nature of the population potentials observed (Fig.1.3) it is apparent that, in magnesium free ACSF, the cells are firing two or more times after a stimulation. This suggests there is an increased availability of neurotransmitter and/or increased postsynaptic sensitivity. The small (12%) increase in the primary potential size suggests there are a greater number of cells firing as well. Thus, adenosine may be required to act on a greater number of cells as well as having to reduce the increased neurotransmission.

The dose response curves to adenosine (Fig.1.4) show a parallel shift to the left or right as the magnesium concentration is raised or lowered. This indicates a "competitive antagonism" of adenosine action by magnesium or, more specifically, a reduction in the percentage inhibition by adenosine due to the increased excitability of the cells. However, when magnesium is omitted altogether,

the slope of the dose response curve is significantly reduced ( $P < 0.001$ , PTT). This suggests that the inhibitory action of adenosine is actively impaired in the absence of magnesium, as opposed to a masking effect as a result of the increased cell excitability. Also, the maximum attainable inhibition by adenosine is reduced so that, in some slices, a 100% decrease of the population potential was not possible even at concentrations as high as 1mM adenosine (Fig.1.6).

Experiments were carried out to determine the relationship between the increased excitability of the slice, apparent by an increase in the evoked population potential size, and the reduced adenosine inhibition. This involved reducing the excitability of the slice without reintroducing magnesium.

During the course of this study three hypotheses were considered as explanations for the reduction in adenosine inhibition. The first, as discussed above, is that the increased cell excitability is masking the action of adenosine. The second is the possibility that there is a requirement for magnesium ions at the adenosine receptor or channel to aid in effective adenosine action. The third hypothesis is that activated NMDA receptor operated channels can directly antagonize the inhibition of synaptic transmission by adenosine. These hypotheses will be referred to during the discussion of these experiments.



#### 1.4.1. Low calcium.

The effect of reducing the extracellular calcium concentration in magnesium free ACSF was probably to reduce synaptic transmission by reducing the calcium available for uptake into synaptic terminals (Dingledine and Somjen, 1981). The primary potential was reduced under these conditions, although the secondary potentials were still apparent. The inhibitory action of adenosine was still significantly reduced despite the reduced synaptic transmission. However, it became apparent that spontaneous activity might be interfering with the evoked potentials (see section 1.4.2. below).

To counteract the spontaneous activity two approaches were tried:

- 1) Removal of the CA2-CA3 region of the slice, the initiation site for spontaneous activity (Wong and Traub, 1983), reduced but did not abolish spontaneous activity. A similar observation has been reported by Traynelis and Dingledine (1989) in CA1 "minislices" where the CA3 had been removed. A reduction of calcium and magnesium from 1.5mM to 1.2mM in the presence of 8.5mM potassium resulted in spontaneous interictal activity that was not normally observed at the higher concentrations of magnesium and calcium. This effect was ascribed to a reduction in membrane surface charge.

- 2) Addition of the GABA agonist baclofen to the slice. The effect of adding a low concentration of baclofen was to reduce the spontaneous epileptiform bursting while having no

observable effect on the evoked population potential. The reason for this differential potency of baclofen is not clear but may be related to the postulated existence of two GABA<sub>B</sub> receptor subtypes (Scott Swartzwelder et al., 1986), one postsynaptic high affinity receptor acting via potassium channels to hyperpolarize cells, the other presynaptic low affinity receptor suppressing synaptic transmission possibly via action at a calcium channel. The net result was to eliminate interference from spontaneous activity. Evoked potentials were reduced in low calcium ACSF, secondary potentials were still apparent but fewer in number and greatly reduced. In most slices, the extracellular calcium had to be reduced to 0.8mM before a significant reduction in potential size was observed. Under these conditions the response to adenosine was still significantly smaller than control levels. This indicates that the effect of adenosine was still impaired in magnesium free ACSF despite the reduced synaptic transmission due to the low calcium. This supports the possibility that a lack of magnesium actively impairs the action of adenosine, as opposed to an indirect effect via increased calcium influx and neurotransmitter release.

#### 1.4.2. Apparent excitatory action of adenosine.

This is a convenient point to digress from discussion of the reduced excitability experiments to consider an effect of adenosine observed during the use of magnesium free/low calcium ACSF. In some experiments the action of adenosine,

when added to a slice bathed in magnesium free/low calcium ACSF, was to cause an increase in the size of the potential. The original hypothesis, that there is a requirement for magnesium at the adenosine receptor, was extended to include the possibility that magnesium is required only at the A<sub>1</sub> adenosine receptor. When binding of adenosine to this receptor is impaired in magnesium free ACSF, activation of the A<sub>2</sub> excitatory receptors becomes apparent. A<sub>2</sub> receptors have been shown to occur in the hippocampus as determined by [<sup>3</sup>H]NECA binding in the presence of R-PIA (Lee and Reddington, 1986) although they are in relatively low density as compared to other regions of the brain such as the striatum (Bruns et al., 1986).

However, use of A<sub>2</sub> specific agonists and A<sub>1</sub> antagonists failed to show a consistent effect that could be attributed to a pharmacological action of the adenosine compounds. When the recording conditions were altered to provide a continuous recording of electrical activity in the slice it became apparent that the "excitation" was a result of inhibition of spontaneous activity by adenosine. Under magnesium free ACSF conditions there was no spontaneous activity apparent when the slice was being stimulated. However, under magnesium free / low calcium conditions, the excitability of the cells appears to be increased so that they fire rapidly during the 10 second interval between stimuli. This action is independent of presynaptic neurotransmitter release and is therefore unaffected by

reduced presynaptic calcium influx and presynaptic adenosine action (see section 1.4.5. below). The reason for the increased excitability is not clear, but may be due to a further reduction in membrane surface charge (McLaughlin, 1971). This increased excitability would "exhaust" the cells, with depletion of presynaptic neurotransmitter reserves and a greater number of cells in the hyperpolarized state that follows immediately after a depolarization. When the slice is stimulated, the total number of cells able to fire is reduced, as is the size of the recorded potential. Adenosine inhibits the spontaneous activity and allows the cells to recover, a change reflected in an increase in the population potential size. This recovery can take up to 5 minutes, which explains the long onset time to response seen in some experiments.

A re-examination of the chart records showed that the increase in potential size with adenosine was never greater than the potential size in magnesium free ACSF, so no true excitation or increase in potential size by adenosine can be claimed. In the slices that exhibited this effect the calcium concentration presumably was not low enough to reduce synaptic transmission or, alternatively, the depression of the evoked potential by spontaneous activity was greater than that imposed by the reduced calcium. In the majority of slices the "excitation" by adenosine was not observed, presumably because the low calcium was reducing synaptic transmission which was the original premise for using low calcium.

#### 1.4.3. Cobalt.

Returning to the reduced excitability experiments, the addition of cobalt to the ACSF reduced the overall excitability of the cells, as was apparent by the reduction in the size of the primary potential and the abolition of the secondary potentials. It also restored the potency of adenosine to control levels. The original premise for adding cobalt was to block a proportion of the presynaptic calcium channels and so reduce neurotransmitter release. The fact that the inhibition by adenosine was restored to control levels when cobalt was added could be explained in two ways. If adenosine requires magnesium for its interaction with its effector mechanism or receptor, then it is also possible that cobalt can effectively substitute for magnesium in this interaction. Reddington et al.(1987) showed that cobalt can effectively substitute for magnesium in enhancing [ $^3\text{H}$ ]CHA binding in rat brain membranes. The enhancement was not as great as with magnesium, but the addition of magnesium did not increase the [ $^3\text{H}$ ]CHA binding further, suggesting that cobalt was acting at the same site as magnesium to enhance CHA binding. Chin and DeLorenzo (1985) also reported that cobalt stimulates the binding of 2-chloro[ $^3\text{H}$ ]adenosine to a novel  $\text{A}_3$  receptor (discussed in chapter 3, p242), while the action of magnesium ions on binding was comparatively low. This receptor is proposed to be associated with calcium channels and, if it exists, it can be postulated that cobalt may restore the action of adenosine by stimulating binding to an  $\text{A}_3$  receptor which is not inactivated by a lack of

magnesium ions. However, this possibility has to be treated as speculative at present, as evidence for this particular  $A_3$  receptor has so far been provided by only one group.

An alternative, that has to be considered in light of the NMDA receptor involvement (see below), is the fact that cobalt has been shown to block selectively the NMDA receptor channel (Riveros and Orrego, 1986; Peet et al., 1986). It could therefore be acting to block calcium influx across NMDA receptor channels which appear to be the principal sites of increased calcium influx under conditions of magnesium free ACSF.

#### 1.4.4. 2-Amino-5-Phosphonopentanoic acid (2-AP5).

The addition of 2-AP5 (50 $\mu$ M) to the hippocampal slice after the introduction of magnesium free ACSF had no effect on the size of the primary population potential and the inhibitory properties of adenosine were not restored. This was taken to indicate that NMDA receptor operated channels were not a major contributing factor to the potential size increase. However, later experiments showed that the situation was more complex than this. Although the size of the secondary potentials was not recorded in all experiments, the effect of 2-AP5 when added in magnesium free ACSF was to reduce, but not abolish, the secondary potentials, while having no effect on the primary potential. Similar experiments by Coan and Collingridge (1987) showed that the effect of 2-AP5 on the CA1 evoked potential in

magnesium free ACSF was dependent on the stimulus size. A low stimulus intensity allowed 2-AP5 to reduce or abolish the primary potential, while a higher stimulus intensity prevented this action and actually produced an increase in potential size at 30-50v. In some slices, notably those that were also exhibiting spontaneous activity, Coan and Collingridge (1987) showed that 2-AP5 was only partially effective in abolishing the secondary potentials. The stimulus size in the present experiments was set to give a population potential 75% of maximum in normal ACSF. This was not altered throughout the experiment. In magnesium free ACSF the effective stimulus intensity may have been sufficient to prevent 2-AP5 from exerting an effect on the primary potential. However, this does not explain why 2-AP5 was more effective in preventing an increase in excitability when it was added prior to the magnesium free ACSF.

The addition of 2-AP5 to the perfusion medium before or with the introduction of magnesium free ACSF prevented the increased excitability and also prevented the loss of adenosine inhibition. This was not the case if 2-AP5 was added after a period of magnesium free ACSF. A possible explanation for this could be that 2-AP5 is locking magnesium into the NMDA receptor operated channel (Sokolovsky and Kloog, 1987), effectively maintaining the magnesium block. Once the magnesium has been washed out, the trapped magnesium ions are still present to keep the NMDA receptor operated channel inactive. If 2-AP5 is added after

magnesium is omitted, 2-AP5 binds to the receptor but is less effective at inactivating the NMDA receptor operated channel once these have been activated by removal of magnesium. The concept of 2-AP5 "freezing" the channels was provided by Sokolovsky and Kloog (1987) to explain the decrease of MK801 and TCP binding in the presence of 2-AP5. These ligands bind to the PCP receptor located in the NMDA receptor operated channel. However, 2-AP5 is an effective inhibitor of magnesium free spontaneous epileptiform bursts (Stanton et al., 1987), so it is able to exert a certain level of antagonism in the absence of magnesium ions. Alternatively, 2-AP5 may be aided in its binding by the presence of magnesium ions, in the same manner as is postulated for the binding of adenosine. This seems unlikely as 2-AP5 is a commonly, and effectively, used antagonist of NMDA receptors in magnesium free ACSF.

Another possibility is that activation of NMDA receptors is required for the initiation of increased neuronal excitability, but secondary mechanisms act to maintain the increased level of excitability. These mechanisms could be via a reduction in GABAergic input or efficacy, an increase in the extracellular potassium concentration or a reduction in neuromodulatory control, possibly by adenosine. Evidence for this possibility can be given by the observation that spontaneous activity invoked by removing the magnesium is frequently difficult to abolish upon reintroduction of magnesium. This has been observed in the neocortex



(Smith et al., 1989), where reintroduction of magnesium after a period of one hour in magnesium free ACSF reduced, but failed to abolish, the spontaneous depolarizations. In some slices, the spontaneous activity was not abolished even when magnesium levels were increased 2.5 times above control. A similar phenomenon was observed in the dentate gyrus (Stratton et al., 1988). This, however, was not the case in the CA1 in the present studies, where reintroduction of magnesium abolished any spontaneous activity and reduced the size of the primary potential. In the study by Coan and Collingridge (1987), addition of magnesium reduced the secondary potentials, although they did not compare the size of the primary potential with control levels, or state whether any spontaneous activity was abolished.

None of these possibilities provide a completely satisfactory explanation for the differential effects of 2-AP5. However, the experiments carried out with MK801 show that the NMDA channel can be blocked after the magnesium has been removed and this restores the action of adenosine. Effectively, MK801 is acting as a replacement for the magnesium ions and blocking the channel, while 2-AP5 only acts at the receptor to close the channel. It can only do this effectively if magnesium, or MK801, is present in the channel. Thus, this scenario is the most likely explanation for the effects observed.

It therefore appears from these experiments that an increase in NMDA receptor operated channel action is responsible for a significant proportion of the increased neuronal excitability.

The increase in the size of the primary population potential when 2-AP5 is present is small compared to the average increase upon going into magnesium free ACSF ( $5.4 \pm 2.5\%$  compared to  $12 \pm 1.6\%$ ). There is also a lack of secondary potentials in the presence of 2-AP5. This suggests that activation of NMDA receptor channels is the principal action responsible for the increased excitability.

The involvement of NMDA channels can also explain the significant decrease in the slope of the dose response curve to adenosine in magnesium free ACSF (Fig.1.4). As discussed in the introduction to this chapter, magnesium ions impose a voltage sensitive block on NMDA receptor operated channels (Nowak et al., 1984) which reduces the contribution of NMDA channels to normal synaptic transmission (Coan and Collingridge, 1986). When magnesium is removed, the NMDA channel is no longer voltage dependent and is readily activated by glutamatergic neurotransmission. When the extracellular magnesium concentration is increased to 4mM or decreased to 0.4mM, there is a parallel shift of the dose response curve which is most likely due to an action of magnesium at voltage sensitive calcium channels and/or a surface charge effect. When magnesium is omitted altogether, NMDA receptor operated channels are also activated,

resulting in a large increase in neuronal excitability. This is the most likely explanation for the large reduction in adenosine inhibition in magnesium free ACSF.

However, the fact that, in some slices, a maximum inhibition of the population potential could not be reached in magnesium free ACSF, even at concentrations of adenosine as high as 1mM (Fig.1.6), suggests that NMDA channel activation not only masks the inhibition by adenosine but can also actively impair the inhibitory action of adenosine.

The final set of experiments carried out in this study lend support to a direct interaction between adenosine and NMDA receptors or channels. The addition of a low concentration of NMDA (5 $\mu$ M) reduced the level of inhibition by adenosine (Table 1.7), while a stable depression of the evoked potential by adenosine could be attenuated by NMDA (Table 1.8). If it were possible to use higher concentrations of NMDA in the presence of adenosine, this may have attenuated the action of adenosine further. However, higher concentrations of NMDA rapidly depressed the evoked potential altogether, presumably by depolarizing and/or increasing the conductance of the CA1 neurones (Collingridge et al., 1983). Lower NMDA concentrations may have been insufficient to overcome the magnesium block completely, acting on only a small number of cells where magnesium block of NMDA channels was weaker. Alternatively the low NMDA concentration may act to reduce the action of adenosine, by proposed mechanisms discussed below, but have

little or no effect on postsynaptic excitability.

The possibility that the reduced adenosine inhibition was simply a result of increased cell excitability due to NMDA was studied by increasing cell excitability without activating NMDA channels. An increased concentration of potassium (8.5mM) was used to increase excitability as this has been shown to act independently of NMDA channel action (Traynelis and Dingledine, 1989) unlike GABA antagonist mediated excitability (Dingledine et al., 1986). In the presence of 8.5mM potassium and 2-AP5 there was a small but significant decrease in the action of adenosine possibly due to the increased excitability. When the 2-AP5 was removed, however, the action of adenosine was severely attenuated in a similar way to that observed in magnesium free ACSF. Thus, despite the increased excitability of the CA1 cells, adenosine was still able to exert an inhibitory action that was only slightly reduced. When the block on NMDA channel activation by 2-AP5 was removed, the voltage dependent NMDA channels would be activated by potassium depolarization of the cell membrane resulting in a reduction in adenosine inhibition. This supports the idea that activation of NMDA channels directly reduces inhibition by adenosine.

#### 1.4.5. Action of adenosine on spontaneous activity.

In contrast to the effect of adenosine on the evoked potential, determination of the action of adenosine on magnesium free induced spontaneous activity demonstrates the potent anticonvulsant properties of adenosine. Both

adenosine and 2-chloroadenosine inhibited epileptiform bursts recorded in the CA3 region with  $IC_{50}$  values of 3.6 and 0.25  $\mu$ M respectively. A similar potency was observed on bursting recorded in the CA1 region. The original idea for looking at adenosine in magnesium free ACSF was to determine the reason for the marked difference in potency of adenosine between the studies by Ault and Wang (1986) and O'Shaughnessy et al. (1988). The potency of adenosine in the present study is comparable to that observed by Ault and Wang (1986) on bicuculline induced bursts in the hippocampal slice and suggests that the difference is not due to the different techniques used to induce the bursts. As suggested by O'Shaughnessy et al. (1988) the disparity is more likely to reflect the lower density of adenosine receptors found in the neocortex (Goodman and Snyder, 1982) or possibly the different neuronal networks in the two brain regions.

The main significance of these results is the comparison between the action of adenosine on magnesium free spontaneous activity and its action on the evoked population potentials. If the action of adenosine is dependent on the presence of magnesium ions, then the anticonvulsant potential of adenosine would be expected to be lower on magnesium free ACSF induced bursts as compared to other models of epileptiform activity. A possible explanation for the difference between adenosine action on spontaneous activity and evoked potentials, is that adenosine is acting at a different site or via a different mechanism to reduce

spontaneous activity. Lee et al.(1984) showed that adenosine can inhibit after-discharges observed during antidromic stimulation of CA1 pyramidal cells in calcium free ACSF, i.e. in the absence of synaptic transmission. This strongly supports a postsynaptic site of action for adenosine.

The A potassium channel, in particular, is thought to control repetitive firing (Segal et al.,1984) and has been shown to be responsible for abolishing repetitive firing upon addition of adenosine. Schubert and Lee (1986) showed that inhibition by adenosine of postsynaptic repetitive firing, observed with antidromic stimulation of CA1 pyramidal cells in low calcium medium, could be abolished if the A channel specific blocker 4-aminopyridine was added. Tetraethylammonium (TEA), a non-specific potassium channel antagonist, had no effect on the anticonvulsant action of adenosine. The A potassium channel is a transient, rapidly inactivated channel and is inactivated at slightly depolarized membrane potentials. The action of adenosine may be to hyperpolarize membranes and bring the A channel into play to abolish spontaneous activity (Schubert and Kreutzberg, 1987). Alternatively, adenosine may act directly on the A channel to activate it and abolish spontaneous activity. This action of adenosine appears to be distinct from the presynaptic inhibitory action of adenosine that is involved in inhibiting evoked, synaptically mediated activation of cells. It also appears to be unaffected by the lack of magnesium ions.

A recent report by Schubert and Mager (1989), looking at changes in extracellular calcium ion concentrations in response to repetitive stimuli, showed that postsynaptic calcium influx into CA1 pyramidal cells was depressed by endogenous adenosine levels in the absence of magnesium. This they took to indicate that postsynaptic receptors were magnesium insensitive, in contrast to presynaptic adenosine receptors that appear to be magnesium sensitive (see below, Schubert and Kreutzberg, 1987).

A further point that should be noted is that spontaneous activity is already regulated by endogenous adenosine. This is apparent from the observation that adding adenosine antagonists to spontaneously depolarizing slices increases the burst firing rate further (Ault and Wang, 1986). Also, endogenous adenosine release has been shown to be increased by adding glutamate to rat cortical slices (Hoehn and White, 1989). An increased glutamate release in magnesium free ACSF might therefore be expected to increase the endogenous adenosine concentration. This may act on the postsynaptic adenosine receptors to reduce significantly the spontaneous bursts, but not abolish them altogether. Under these conditions a very low concentration of added adenosine would be sufficient to abolish the bursts altogether. This would enhance the potency of adenosine on spontaneous activity, but not evoked potentials which appear to be regulated mainly by presynaptic magnesium sensitive adenosine action.

#### 1.4.6. Possible explanations for the reduction in adenosine inhibition.

The most straightforward explanation for the effects of magnesium on adenosine reported in this chapter is that NMDA channel activation increases the excitability of the pyramidal cells and, as a result, reduces the apparent inhibition by adenosine by masking its action. Increased postsynaptic NMDA channel activation of CA3 pyramidal cells could increase presynaptic neurotransmitter release from synapses in the CA1 region as it is the collateral projections from the CA3 cells that form the presynaptic terminals being studied in this system.

Alternatively, if it is assumed that NMDA channels exist on presynaptic nerve terminals (see below), presynaptic calcium influx may occur along these channels and effectively reduce the presynaptic action of adenosine by providing a second route of calcium influx. This would also explain why adenosine is unable to abolish synaptic transmission totally in magnesium free ACSF as calcium would be able to enter via presynaptic NMDA channels regardless of the concentration of adenosine applied. The experiments where the extracellular calcium concentration was lowered to reduce synaptic transmission, failed to restore adenosine inhibition to control levels. However, if calcium influx does occur across presynaptic NMDA channels, then a reduction in calcium availability would not prevent calcium influx across the NMDA channel and adenosine action would still be unable to reduce the calcium influx. It is also



possible that the affinity of the NMDA channel for calcium may be greater than that of the adenosine regulated calcium channels, resulting in calcium influx occurring preferentially across the NMDA channel.

It is therefore possible to ascribe all the results of this study to an increase in calcium influx across NMDA receptor operated channels, but only if the existence of presynaptic NMDA channels is assumed. However, the results could equally well be ascribed to a direct inhibition of adenosine action by NMDA channel activation. A requirement for magnesium ions for adenosine action may also be involved. Both these possibilities will be discussed in detail below.

#### 1.4.6a. A magnesium requirement for the action of adenosine.

From the evidence of the 2-AP5 experiments, it appears that the action of NMDA receptor channels significantly inhibits the action of adenosine. It should be noted, however, that the percentage inhibition by adenosine in magnesium free ACSF with prior addition of 2-AP5 is still significantly less than control ( $p < 0.05$ , Table 1.5.). This may be due to a small effect from the reduced surface charge. However, the original hypothesis, that adenosine requires the presence of magnesium ions for it to exert an inhibitory action, is still of interest not least because of the large body of evidence that supports this possibility. These studies, and the possible ways by which magnesium may interact with adenosine are discussed below.

(i) Magnesium requirements of adenosine binding.

In ligand binding studies there is good evidence for a regulation of adenosine binding by the ions present in the extracellular fluid. Goodman et al.(1982) originally demonstrated an effect of divalent cations on adenosine receptor binding. Using guinea-pig brain membranes they were able to show an enhancement of [ $^3\text{H}$ ]CHA binding with magnesium, manganese and calcium ions. Manganese was the most potent, maximal enhancement of binding occurring at 0.3mM, while magnesium and calcium effects were maximal at 1mM. The effect of the cations is to increase the receptor affinity as well as increase the number of binding sites, as determined by Scatchard analysis. Pretreatment of the membranes with EDTA resulted in a 50% loss of receptor binding, while addition of the divalent cations enhanced binding by 150-170%. Without EDTA pretreatment the cations only increased binding by 40%. This indicates that membrane bound ions are aiding in adenosine receptor binding.

Gavish et al.(1982) showed that, if bovine brain membranes were solubilized to isolate adenosine receptors, the enhancement properties of magnesium ions on adenosine binding were lost. In a study by Linden et al.(1987) the requirement of magnesium for effective ligand binding was decreased in solubilized receptors. Either way, the presence of magnesium or a suitable divalent cation is required on, or adjacent to, the membrane located adenosine receptor for maximal binding to take place.

Linden et al.(1987) used rat brain cortical membranes to study the effect of  $\text{MgCl}_2$  and  $\text{NaCl}$  on  $^{125}\text{I}$ -ABA (aminobenzyladenosine) binding. Binding to membranes pretreated with 10mM EDTA was shown to be  $\text{MgCl}_2$  dependent with concentrations of 2-5mM  $\text{MgCl}_2$  required for maximal binding. The addition of  $\text{NaCl}$  at concentrations between 30 and 2000mM reversed the  $\text{MgCl}_2$  dependent binding with an  $\text{EC}_{50}$  of 200mM.  $\text{NaCl}$  in the absence of  $\text{MgCl}_2$  had no effect, binding being virtually zero. It is thought that monovalent cations, such as  $\text{Na}^+$ , can convert adenosine receptors to a medium affinity receptor state while the presence of  $\text{Mg}^{2+}$  ions maintains the receptor in a high affinity state (Green, 1984). Conversely, addition of guanine nucleotides and NEM converts the receptor to a low affinity state, possibly by disassociating the subunits of the G-proteins and thus uncoupling the receptors (Asoni and Ogasawara, 1986).

In adipocyte plasma membranes Yeung et al.(1985) found a slightly different situation, where addition of magnesium to the ligand binding experiments decreased  $[^3\text{H}]\text{CHA}$  binding, as did the addition of GTP. Scatchard analysis indicated this was as a result of decreased receptor number. However, if the two were added together,  $[^3\text{H}]\text{CHA}$  binding was greatly enhanced. The addition of NEM abolished this effect and also the effect of magnesium alone, but had no effect on the GTP reduction in binding. The authors proposed that a specific magnesium site exists that is required for the interaction of the adenosine receptor with its effector site, possibly

at the site of receptor coupling with a G-protein. They also reported that sodium ions, when added with magnesium, enhanced binding which was additive with the effects of low concentrations of GTP.

The effects observed by Yeung et al.(1985) are in contrast to those reported by Goodman et al.(1982) and Linden et al.(1987). No obvious reason for this is apparent. It is possible that there are differences between CNS membranes and adipocyte membranes. Alternatively, the fact that the membranes were not pretreated with EDTA in the study by Yeung et al.(1985) may suggest they were looking at an indirect effect of receptor affinity and availability as determined by interactions of magnesium and GTP on the intracellular side of the membrane, while Goodman et al.(1982) and Linden et al.(1987) were looking at an effect of divalent cations on direct binding of adenosine to its receptor, i.e. an extracellular effect only apparent when the membrane bound cations are removed by EDTA. The conclusion of Yeung et al.(1985) presupposes an intracellular site of action for the interaction of the divalent cations with adenosine effector mechanisms, as these biochemical processes are known to occur on the inside of the plasma membrane. In an intact system it would be difficult to conceive of a situation where removal of extracellular ions would be sufficient to reduce the ions available intracellularly for enzyme interactions, particularly as the loss of adenosine potency is apparent after 10-20 minutes in zero magnesium ACSF.

(ii) Biochemical requirements for magnesium.

The biochemical requirements for magnesium ions also need to be looked at as a possible site for interactions of magnesium with adenosine. Magnesium acts as a cofactor for a number of enzymes, in particular it forms a complex with ATP which is important as a cofactor in many biochemical reactions (Devlin, 1982). The enzyme 5'-nucleotidase is responsible for converting AMP to adenosine. This enzyme is inhibited by the presence of ATP and ADP, but not by ATP-Mg or ADP-Mg. As the nucleotides readily complex with  $Mg^{2+}$  ions, inhibition of 5'-nucleotidase is kept under control (Arch and Newsholme, 1979). As can be seen in Fig.2, p27, 5'-nucleotidase forms a substrate cycle with adenosine kinase. If the magnesium ion concentration is reduced, this may cause an imbalance in the biochemical homeostasis, resulting in an increase in uncomplexed ATP and ADP. The increased inhibition of 5'-nucleotidase would reduce the formation of adenosine from AMP, while adenosine deaminase, which is not affected by magnesium and has a greater  $K_m$  for adenosine than adenosine kinase (Arch and Newsholme, 1979), would rapidly break down the available adenosine.

Other enzymes in the adenosine metabolic pathway that require magnesium for optimal activity are hypoxanthineguanine phosphoribosyltransferase (HGPRTase) and adenine phosphoribosyltransferase (APRTase) (Devlin, 1982). A reduction in either of these enzymes would result in a decrease in AMP levels, the principal substrate for

adenosine formation. Thus, in theory at least, a reduction in magnesium levels at the biochemical level could lead to an imbalance in adenosine metabolism, leading to an overall reduction in adenosine availability.

In the context of the present experiments it is possible that an imbalance in adenosine metabolism could increase adenosine uptake and catabolism such that the added adenosine is taken up and broken down, reducing its action at the receptor. However, the principal argument against this is that 2-chloroadenosine, which is not a substrate for the adenosine uptake system (Clanachan et al., 1986), is also affected by the lack of magnesium ions. Also, as with the ligand binding studies, it is unlikely that magnesium ions, particularly intracellularly, would be sufficiently reduced in the 10-20 minutes it takes for the adenosine inhibition to be reduced, to significantly affect the concentrations of nucleotide-Mg complexes, or the other enzymes in the metabolic pathways.

The concept of an interaction of magnesium ions with adenosine receptors to promote adenosine binding should not be dismissed altogether. Ligand binding studies repeatedly show that magnesium ions enhance adenosine receptor agonist binding. Similarly, other receptors have also been shown to be affected by the extracellular ion concentration (Baudry and Lynch, 1979; Tsai and Lefkoistz, 1978), and the levels of endogenous metal ions can affect neuronal functions

(Davidson and Ward, 1988; Chung and Johnson, 1983). It is therefore not inconceivable that a ubiquitous neuromodulator such as adenosine might be influenced in its actions by the endogenous levels of ions, thus explaining some of the changes in neuronal functions and excitability. A regulatory role for magnesium ions could occur at the extracellular adenosine receptor with a specific site on the receptor requiring a magnesium ion for the effective binding of adenosine to take place. Alternatively, there may be a requirement for magnesium ions at the adenosine associated channel itself, in a similar manner to the NMDA channel.

#### 1.4.6b. Inhibition of adenosine action by NMDA receptor channel activation.

In the present set of experiments the reduction in adenosine inhibition can more readily be explained by an increase in NMDA receptor operated channel activity. This presents the possibility that activation of NMDA channels may impair the action of adenosine directly, as opposed to a simple masking effect due to increased cell excitability.

An interaction between NMDA receptor operated channels and the adenosine effector mechanism is a possibility, although the published literature provides only sparse and indirect evidence.

An abstract by Stratton et al.(1988) reported a total loss of the inhibitory action of adenosine on rat dentate gyrus population potentials when magnesium was omitted from

the ACSF. This effect could be prevented by 2-AP5 if added before, but not after, the period of magnesium free ACSF. Reintroduction of magnesium failed to restore the effect of adenosine unless the slice was not stimulated during the magnesium free period, indicating that activation of NMDA channels was needed to block the action of adenosine.

A recent report that may be linked to an effect of adenosine on NMDA channels was a study of the effect of a new adenosine antagonist on hippocampal CA3 neurones. Alzheimer et al.(1989) reported that addition of the A<sub>1</sub> specific adenosine antagonist, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), resulted in spontaneous depolarizations of the CA3 cells that persisted even after 2-3 hours of washout of the drug. The addition of adenosine induced a hyperpolarization of the cells, apparent during intracellular recording. This action was reduced during addition of DPCPX but was restored upon washout, indicating the DPCPX was not irreversibly bound to the adenosine receptors. Similarly, addition of bicuculline produced an increase in the number of spontaneous bursts, indicating that the irreversible excitability was not due to reduced GABAergic activity. One possibility that was not explored was that a reduction in purinergic tone may result in activation of NMDA channels which, once activated, could not be deactivated by restoration of the action of adenosine.



Interesting studies by Schubert and colleagues have demonstrated a similar loss of effect of adenosine on synaptic calcium influx in magnesium free ACSF (Schubert and Kreutzberg, 1987; Schubert and Mager, 1989). These studies involved the use of calcium ion sensitive electrodes to measure the reduction in extracellular calcium upon rapid stimulation of the S. radiatum fibres to the CA1 pyramidal cell dendrites of the hippocampal slice. The calcium sensitive electrode was placed on the border of the S. radiatum and the S. pyramidale to record both presynaptic calcium influx and postsynaptic influx into the pyramidal cells. In low calcium (0.2mM), high magnesium (4mM) ACSF, synaptic transmission is blocked and pre- and postsynaptic calcium influx, theoretically, can be differentiated with orthodromic or antidromic stimulation. Under these conditions, adenosine (40 $\mu$ M) gave a 25% drop in the calcium signal during orthodromic stimulation, while antidromic stimulation produced only a 7% decrease. With normal levels of calcium (2mM) and magnesium (2.4mM) synaptic transmission was present and orthodromic stimulation resulted in a decrease in the calcium signal, representative of calcium uptake into presynaptic terminals and postsynaptic cells. Addition of 20 $\mu$ M adenosine reduced the drop in the calcium signal, indicating a decrease in calcium influx. Omitting magnesium from the ACSF led to a large increase in calcium influx and the addition of 20 $\mu$ M adenosine had no effect on the calcium influx in magnesium free ACSF. This observation supports the idea that the loss of adenosine inhibition in

the present experiments is not simply a masking effect but a real loss of the ability of adenosine to inhibit presynaptic calcium influx in magnesium free ACSF.

In the report by Schubert and Kreutzberg (1987) the addition of theophylline (50 $\mu$ M) under the low magnesium conditions resulted in a further increase in calcium influx above that caused by magnesium free ACSF. The addition of 2-AP5 blocked a large proportion of the increased calcium influx seen in magnesium free ACSF, while the addition of theophylline in the presence of 2-AP5 did not increase calcium influx further, indicating that endogenous adenosine is antagonizing an increase in calcium influx elicited by NMDA receptors.

Thus, it appears that endogenous adenosine can impose a tonic inhibition on activation of NMDA receptor channels, but this action is lost when NMDA channels are activated by removing the magnesium ion block, as added adenosine is unable to reduce the 2-AP5 sensitive calcium influx. A proportion of NMDA channels might still be expected to be blocked by magnesium as it is unlikely that magnesium free ACSF will wash out the magnesium from all the NMDA channels. When theophylline is added the endogenous adenosine inhibition on the remaining NMDA channels may be removed and the calcium influx thus increased. The addition of 2-AP5 blocks the enhancing action of theophylline on calcium influx supporting the idea of an action at NMDA receptor operated channels. This is the interpretation of the results suggested by Schubert and colleagues.

However, the possible limitations of this technique should be considered. The measured decrease in extracellular calcium cannot safely be attributed solely to influx into a specific cell type. In particular, glial cells can take up calcium (Hertz et al., 1989), and basket cells and other neuronal fibres may also take up calcium in response to the high frequency electrical stimulation. The evidence given by Schubert and colleagues to support the idea that calcium influx is into neuronal cells and not into other cells is the fact that anti- and orthodromic stimulation gives markedly different changes in the calcium signal, suggesting that calcium uptake is into different cell types or across different numbers of channels as might occur between pre- and postsynaptic regions. Similarly, changes due to experimental manipulations vary between the two forms of stimulation, and the size of the calcium signal also correlates well with the size and quality of the evoked potentials.

If the assumptions associated with this technique are accepted then these experiments provide evidence for a presynaptic action of adenosine on calcium influx and a magnesium sensitive component of adenosine action that may involve an interaction with NMDA receptor channels.

From the experimental evidence discussed above a postulated mechanism, for the loss of adenosine inhibition observed in the present experiments, can be put forward.

Activation of NMDA channels in magnesium free ACSF may reduce the inhibitory action of adenosine, not simply due to increased excitability, as adenosine is unable to produce a 100% reduction in the evoked population potential in the absence of magnesium (Fig.1.6), but by actively impairing the functional effects of adenosine.

An interaction between adenosine and NMDA receptor mediated events would, presumably, require the receptors to exist in close proximity to each other. NMDA receptors are assumed to exist predominantly on the postsynaptic membrane, acting in response to presynaptic amino acid release (Fagg et al.,1986), while the adenosine receptors in this study are assumed to exist presynaptically. However, there is limited evidence for the existence of presynaptic NMDA receptors which could interact directly with presynaptic adenosine receptor sites. Collingridge et al.(1983) observed that high doses of iontophoretically applied NMA produced a 2-AP5 sensitive reduction in the presynaptic fibre volley of CA1 evoked potentials. This suggests that either NMDA receptors exist presynaptically or that postsynaptic NMDA activation can reduce presynaptic activity. Errington et al.(1987) showed that 2-AP5 at high concentrations (50-100 $\mu$ M) could reduce calcium dependent, potassium evoked [ $^{14}$ C]glutamate release from rat dentate gyrus slices. This indicated that NMDA receptors could promote glutamate release from, presumably, presynaptic terminals, suggesting the existence of presynaptic NMDA receptors.

Similarly, Arvin et al.(1986) showed that NMDA can increase the level of intracellular free calcium of rat brain synaptosomes as determined by quin 2. This increase was blocked by magnesium and 2-AP5. They argued that this indicated a presynaptic site of action for NMDA.

Thus it can be hypothesised that a population of presynaptic NMDA receptor operated channels act to reduce the presynaptic action of adenosine. This may occur via a number of speculative mechanisms.

Adenosine and NMDA may act at the same presynaptic calcium channel with opposing actions on calcium influx. However, the NMDA receptor operated channel is directly regulated by magnesium ions and is normally inactive during synaptic transmission (Coan and Collingridge, 1987), while exogenous adenosine is able to block synaptic transmission completely and so presumably acts at a calcium channel that plays a major role in synaptic transmission. It is possible that the NMDA and adenosine receptor associated channels are closely associated and activation of the NMDA associated channel is able to reduce the action of adenosine on its channel. Stratton et al. (1988) suggested that protein kinase C activation may be involved in inhibition of adenosine action by NMDA channel activation. Addition of phorbol esters, which activate protein kinase C, to hippocampal slices blocked the action of adenosine on dentate gyrus potentials in a similar manner to that observed when NMDA channels were activated.

Another possibility is a direct or allosteric interaction at a common binding site. However, there is no evidence, to my knowledge, of an interaction between adenosine and NMDA in binding studies. Studies of direct NMDA binding provide only poor results possibly due to the transient nature of NMDA binding to this receptor (Foster and Fagg, 1984). The use of radiolabelled NMDA antagonists or [ $^3\text{H}$ ]L-glutamate binding in the presence of NMDA antagonists can be used to distinguish NMDA binding sites indirectly (Cotman et al., 1987). Studies of NMDA on adenosine binding may also prove of interest. However, the fact that 2-AP5 had no effect on adenosine inhibition or cell excitability if added after magnesium had been removed suggests that it is the open NMDA calcium channel, rather than the receptor, which affects the inhibitory action of adenosine.

Alternatively, postsynaptic NMDA receptor activation may be able to affect presynaptic adenosine action by some, as yet undefined, mechanism (Collingridge and Bliss, 1987). Current research into the mechanisms of long term potentiation (LTP) is focussing on specific proteins (Fazeli et al., 1988) and arachidonic acid (Lynch et al., 1989) that are released when LTP is triggered. These are hypothesized to act as retrograde messengers that are released from a postsynaptic site and pass to the presynaptic membrane to regulate neurotransmitter release. It is possible that a similar system exists that exerts an inhibitory action on presynaptic adenosine receptors when postsynaptic NMDA

receptor channels are activated. Indeed, LTP may be one system where a regulation of adenosine receptor action may be of benefit (see general discussion), and the retrograde messengers being studied in relation to LTP may also prove to act on adenosine.

Finally, a postsynaptic activation of NMDA receptors might also affect postsynaptic adenosine action, possibly reducing or overriding the hyperpolarization of the postsynaptic membrane by adenosine. However, the fact that adenosine is effective against magnesium free spontaneous depolarizations, presumably by a postsynaptic action as discussed earlier, argues against this possibility.

In this discussion an attempt has been made to discuss in detail the possible mechanisms by which a reduction in extracellular magnesium might influence the inhibitory action of adenosine. An inhibition of adenosine action by NMDA receptor activation is an interesting possibility, although one that is so far supported by indirect evidence only. The possible benefits that an interaction of this type might have in vivo will be discussed in the general discussion.

## CHAPTER 2



## CHAPTER 2

### INTERACTIONS BETWEEN ADENOSINE AND THE DIHYDROPYRIDINE CALCIUM CHANNEL BLOCKERS.

#### 2.1. INTRODUCTION

The action of adenosine in reducing synaptic transmission is strongly associated with a reduction in neurotransmitter release which involves, in some way, a regulation of calcium mobilization at the site of neurotransmitter release. This action has led to adenosine analogues being referred to as "calcium antagonists" (Silinsky, 1981). The other established organic calcium channel antagonists, the phenylalkylamines, dihydropyridines and benzothiazepines, are potent blockers of calcium influx into muscle and cardiac tissue but appear to have only weak or undetectable effects on neuronal calcium channels. As discussed in the main introduction, the dihydropyridines, in particular, bind to multiple high affinity binding sites in the central nervous system (Gould et al., 1982) but appear to have no effect on calcium influx (Daniell et al., 1983) or only act on a small population of calcium channels in neuronal tissue (Thayer et al., 1986).

However, a number of studies, mainly involving ligand binding, have reported an interaction between adenosine receptor binding and the dihydropyridines. This reveals a potent displacement of adenosine receptor ligands by the dihydropyridines, in particular nifedipine and BayK 8644

(Murphy and Snyder, 1983; Hu et al.,1987). On the other hand, the few functional studies that have been carried out show a marked enhancement of the effect of adenosine on neuronal firing by nifedipine (Phillis et al.,1984). This appears to be related to a reduction in adenosine uptake in the presence of the dihydropyridines (Phillis et al.,1984; Morgan et al.,1987). However,the action of R-PIA on noradrenaline release from hippocampal slices was reduced by BayK 8644 but not nifedipine. (Fredholm et al.,1986).

These interactions, between adenosine and the dihydropyridines, may explain some side effects of the dihydropyridines in clinical use. Nifedipine is of particular interest. This drug is used clinically to treat chronic angina and is also effective in the treatment of hypertension, achalasia (chronic constriction of the stomach cardiac sphincter muscle), and Raynauds syndrome (prolonged vasoconstriction of the arterioles in the extremities). The prophylactic effects on these conditions occur via a block of slow calcium channels, resulting in smooth muscle relaxation. However, the use of nifedipine has a much higher incidence of side effects than the other established calcium blockers, verapamil and diltiazem (Swanson and Green, 1985). In particular, the use of nifedipine to treat angina has been reported to increase anginal pain and even induce myocardial ischaemia in a small percentage of cases in clinical studies (Stone et al.,1983; Boden et al.,1985). The possible cause of this detrimental side effect has been

studied in animal models and suggests that nifedipine interferes with the regulation of coronary blood flow by adenosine. Merrill et al.(1983), using an in vivo canine heart preparation to study coronary blood flow, showed that coronary vasodilatation induced by added adenosine could be significantly attenuated by nifedipine. Adenosine is released in response to a brief period of myocardial ischemia induced by coronary artery occlusion. The addition of nifedipine inhibited the post ischaemic blood flow increase (reactive hyperaemia) indicating that nifedipine also blocks the action of endogenous adenosine. A similar effect on reactive hyperaemia was observed on rat cerebral blood flow (Phillis et al.,1986).

A recent study by Borea et al. (1989) has also demonstrated the interaction between the dihydropyridine calcium channel agonist BayK 8644 and adenosine analogues in guinea pig atria. The  $A_1$  adenosine receptor analogues R-PIA, CHA and NECA, but not the  $A_2$  agonist CV1808, reduced the contractile tension and the frequency of spontaneous beating of the atria while BayK 8644 enhanced these effects. However the adenosine analogues antagonized the positive inotropic effects of BayK 8644 when added together. Thus, adenosine analogues can interact with both dihydropyridine calcium agonists and antagonists. This appears to occur at the  $A_1$  receptor, at least in the heart. [ $^3$ H]nitrendipine binding studies indicated this interaction was not direct competitive inhibition at the dihydropyridine receptor.

Other effects of nifedipine, both beneficial and detrimental, can also be attributed to the effects on adenosine. Tourette's syndrome, thought to be a disorder of purine metabolism in the CNS (Van Woert et al.,1977), has been successfully treated with nifedipine. As nifedipine itself is relatively inactive in central neuronal preparations (Phillis et al.,1984; Daniell et al.,1983; this chapter) it is possible that nifedipine is potentiating the action of endogenous adenosine to alleviate the symptoms of Tourette's syndrome.

It is not clear what interactions are occurring in these systems. If nifedipine is acting to reduce the action of adenosine, possibly by displacing it from its receptor, then this would explain the observations in the heart preparation where nifedipine blocks the vasodilatory action of adenosine in coronary arteries (Merrill et al.,1983). However, if nifedipine is acting by inhibiting adenosine uptake and thereby increasing its potency, this would explain any beneficial effects it may be having on conditions where purine metabolism is impaired, such as Tourette's syndrome. There may also be a functional difference between the peripheral and central actions of nifedipine on adenosine. As nifedipine is also known to affect ATP action (Bulloch and McGrath, 1988; Stone, 1981b) and is thought to be active mainly on peripheral neurones, it is possible that the action of nifedipine in peripheral tissues is being elicited via an action on ATP, rather than adenosine, while an

adenosine / nifedipine interaction, different from that exerted on ATP, is involved in the CNS.

These proposed interactions of the dihydropyridines with the adenosine system are still very speculative, particularly in relation to the prophylactic effects of these drugs in certain conditions. However, these interactions with adenosine will hopefully lead to a better understanding of the actions of adenosine and possibly may provide a route towards utilizing drugs that alter the action of adenosine for clinically beneficial purposes.

The initial aim of this study was to try and determine a common effect between adenosine and the dihydropyridines on calcium associated events. As nifedipine has been shown to reduce electrically induced epileptiform activity in vivo (Meyer et al., 1986), the action of nifedipine on epileptiform bursts was determined. Also, as no published studies have reported the effect of adenosine analogues and the dihydropyridines on electrophysiological population potential studies, it was decided to try and resolve the rather confusing, and apparently conflicting, information by looking at the effect of a range of adenosine analogues and calcium channel blockers on evoked population potentials in the hippocampal slice.

## 2.2. MATERIALS and METHODS.

### 2.2.1. Compounds.

Dihydropyridines were a gift from Bayer. BayK 8644 was a gift from Dr.Franckowiak.

The dihydropyridines were dissolved in ethanol as a 10mM stock, and protected from light. Fresh stock solutions were made up each day.

### 2.2.2. Methods.

The preparation of hippocampal slices, and the recording of orthodromic evoked population potentials from the CA1, or spontaneous activity from the CA3, were carried out as described in chapter 1.

Dihydropyridines and adenosine analogues were bath applied via the gravity feed system.

All experiments were carried out under sodium lamp lighting to protect the light sensitive dihydropyridines.

All results are expressed as mean  $\pm$  standard error of the mean (sem) unless stated otherwise. Statistical significance was determined using a paired sample t-test on the Oxstat statistical analysis computer programme.

## 2.3. RESULTS.

### 2.3.1. The action of nifedipine on spontaneous epileptiform activity.

The ability of the dihydropyridine, nifedipine to block calcium channels in the hippocampal slices was assessed by determining its effect on epileptiform activity induced by magnesium free ACSF in the CA3 region of the hippocampal slice. (Fig.2.1.).

Initial results were very erratic due to the sensitivity of the bursts to small changes in ACSF flow rate and temperature. This was kept to a minimum by using the same perfusion tube for control and test solutions and ensuring the separate beakers were at the same height in the gravity feed system. Despite this, small variations were seen when the solutions were changed even when no drug was present. This explains the variability between the different nifedipine concentrations. No significant or consistent effect on the burst frequency was observed with nifedipine.

Single experiments were carried out with BayK 8644, nitrendipine and nimodipine (data not shown). No significant effects on magnesium free ACSF induced spontaneous activity was observed with any of the dihydropyridines.

Fig. 2.1. The effect of nifedipine on spontaneous activity induced by magnesium free ACSF

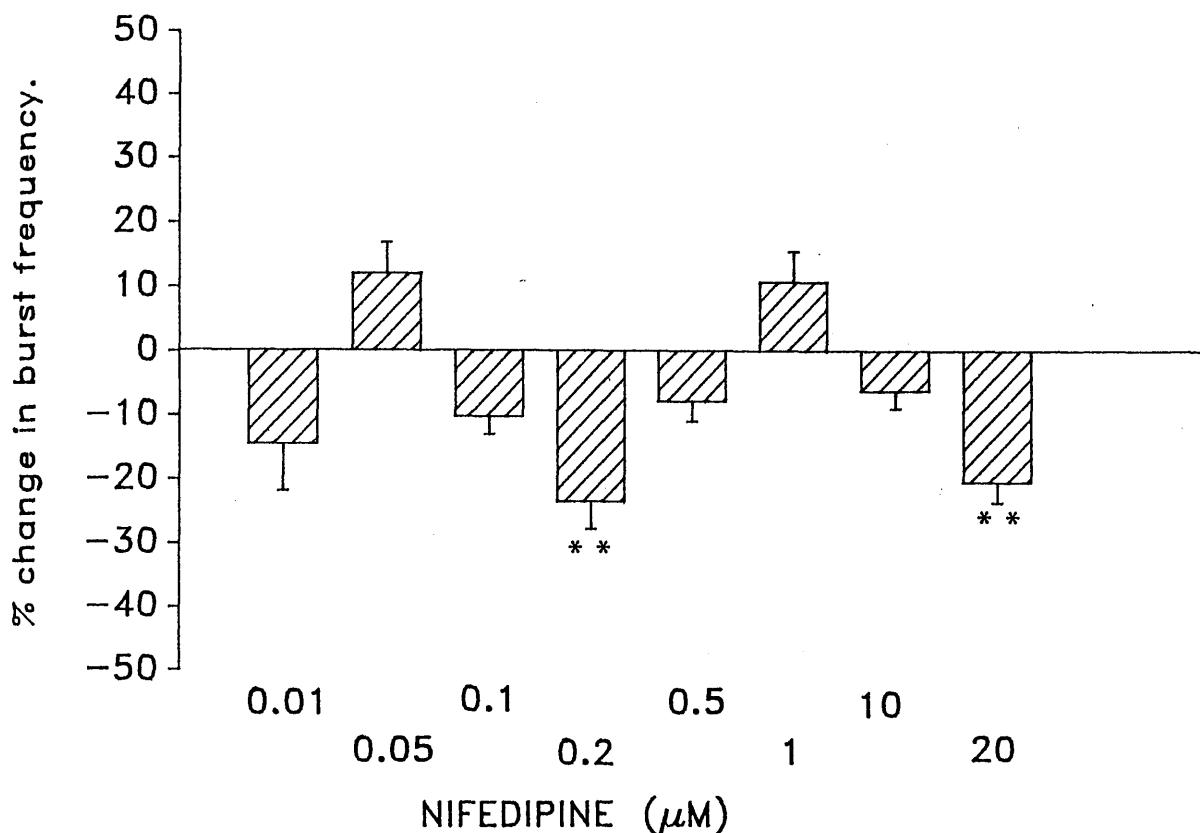


Fig.2.1 Bars represent the percentage change in the burst frequency with respect to the frequency immediately before addition of nifedipine. Burst frequency was determined as bursts per minute measured over a 10 minute period. Points are mean  $\pm$  sem of 4-8 determinations. \*\*=  $p < 0.05$  (PTT).



2.3.2. The action of dihydropyridines on the evoked CA1 population potential: Interactions with adenosine and adenosine analogues.

The addition of nifedipine at concentrations up to 50 $\mu$ M had no effect on the CA1 evoked population potential. However, when adenosine was added in the presence of nifedipine, there was a significant potentiation of the inhibitory action of adenosine on the population potential.

The action of adenosine and related analogues on evoked CA1 population potentials was determined in the presence of various calcium channel blockers, in particular the dihydropyridines.

The two compounds that significantly affected the action of adenosine and its analogues were the dihydropyridines nifedipine and BayK 8644, a calcium channel antagonist and agonist respectively.

Dose response curves to nifedipine and BayK 8644 were determined (Figs. 2.2-2.3). Control responses to adenosine and 2-chloroadenosine were determined prior to the addition of the dihydropyridines which were allowed to perfuse for 20 minutes. The effect of the adenosine compounds in the presence of the dihydropyridines was then determined. A 30 minute washout period was allowed between each dihydropyridine concentration. At the end of the experiment, the dihydropyridines were allowed to wash out for up to 2 hour. However, a return to control adenosine responses was not observed in any of the experiments.

Fig. 2.2. The effect of nifedipine and BayK 8644 on the inhibitory action of 2-chloroadenosine.

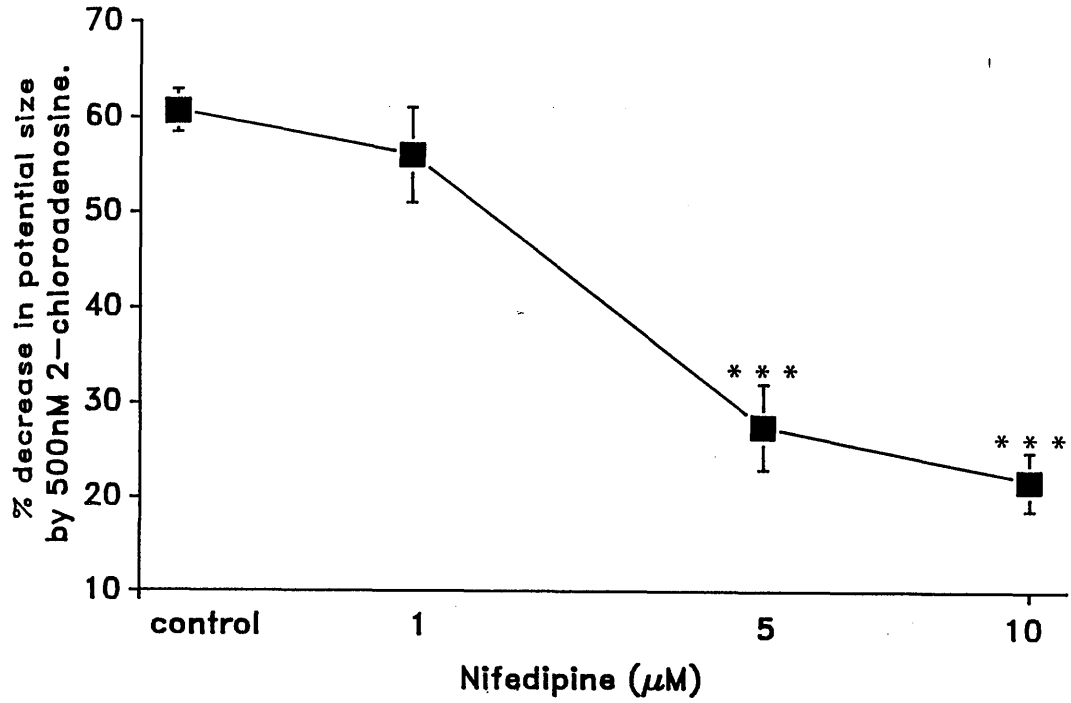
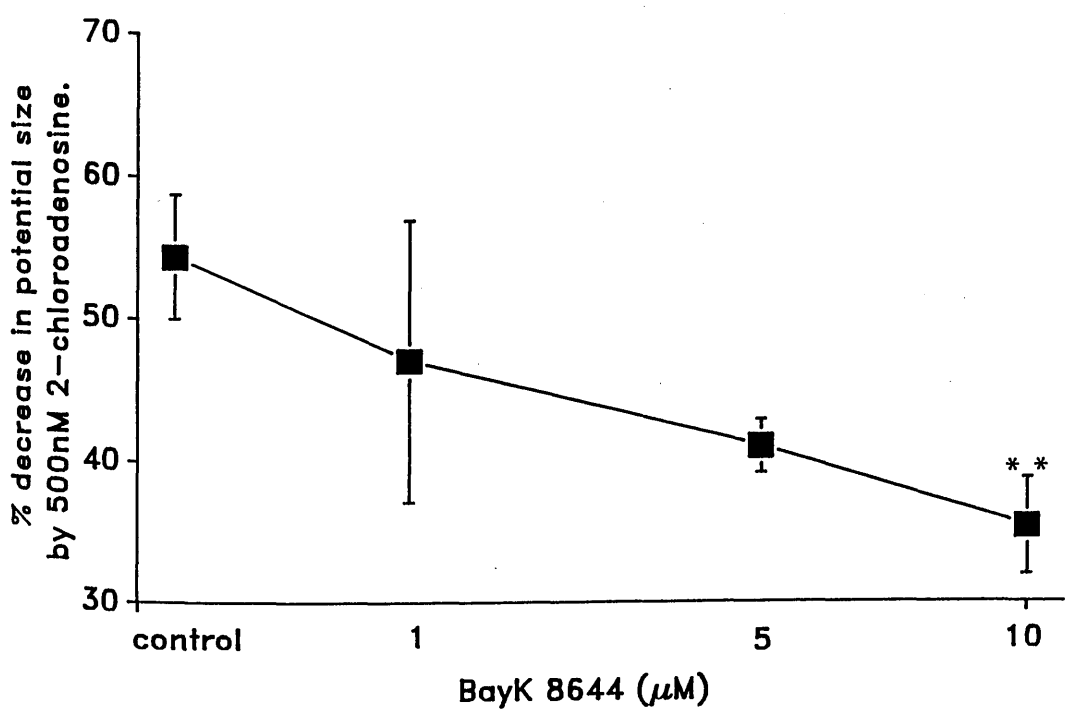


Fig 2.2 a. Effect of nifedipine on the percentage decrease of the CA1 population potential by 0.5 $\mu\text{M}$  2-chloroadenosine. Points are mean  $\pm$  sem of 8-14 determinations. \*\*\*=  $P < 0.001$  (PTT).



Figs 2.2 b. Effect of BayK 8644 on the percentage decrease of the population potential by 0.5 $\mu\text{M}$  2-chloroadenosine. Points are mean  $\pm$  sem of 8-14 determinations. \*\*=  $P < 0.05$  (PTT).

Fig. 2.3. The effect of nifedipine and BayK 8644 on the inhibitory action of adenosine.

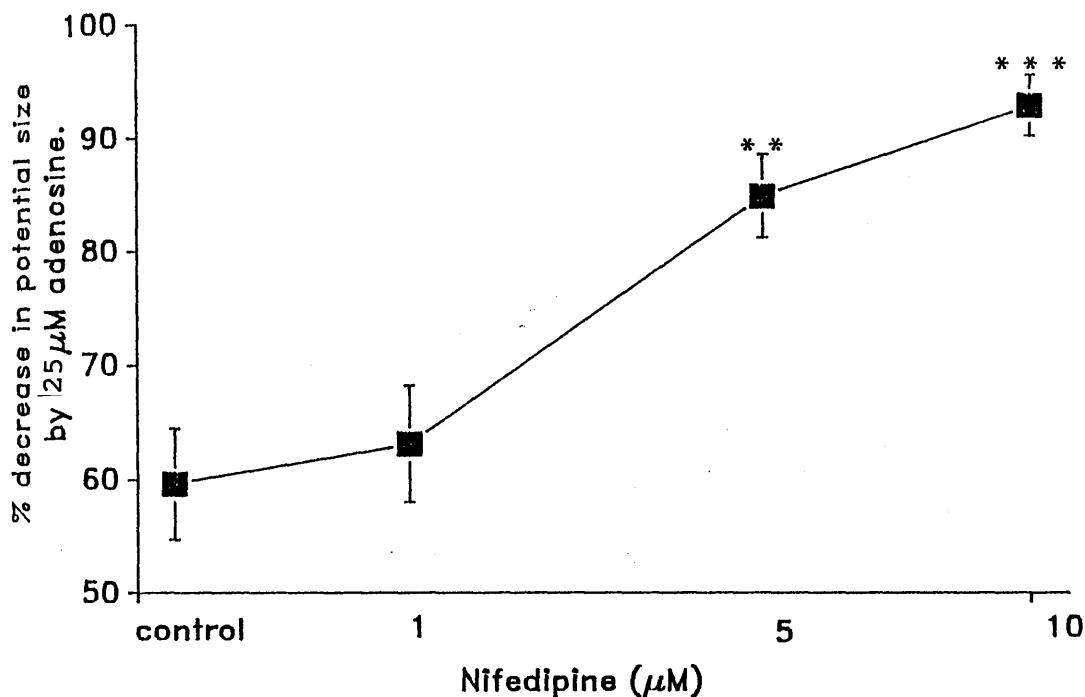
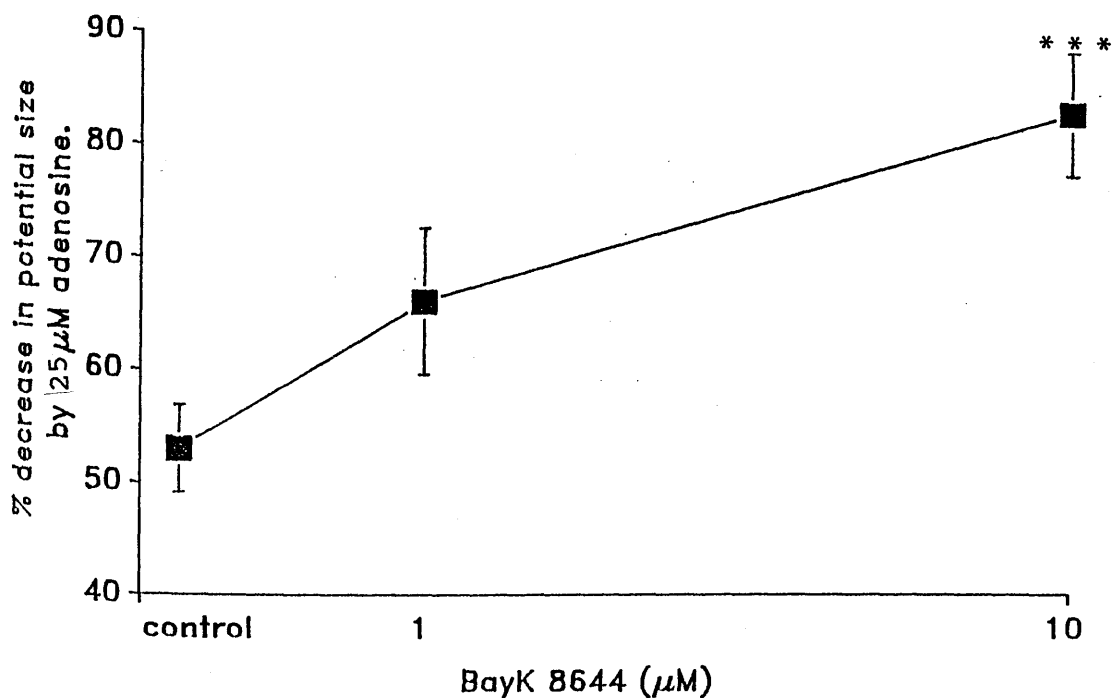


Fig 2.3 a. Effect of nifedipine on the percentage decrease of the population potential by  $25\mu\text{M}$  adenosine. Points are mean  $\pm$  sem of 8-14 determinations. \*\*=  $P < 0.05$ , \*\*\*=  $P < 0.01$  (PTT).



Figs 2.3 b. Effect of BayK 8644 on the percentage decrease of the population potential by  $25\mu\text{M}$  adenosine. Points are mean  $\pm$  sem of 8-14 determinations. \*\*\*=  $P < 0.01$  (PTT).

Responses to cyclohexyladenosine (CHA) and R-phenylisopropyladenosine (R-PIA),  $A_1$  specific agonists, and to N-ethylcarboxamidoadenosine (NECA), an agonist with partial affinity for the  $A_2$  receptor, were also determined. The use of CHA and R-PIA as agonists was complicated by the long onset and longer washout periods of these drugs. The length of time required for a concentration of compound that gave a submaximal inhibition to attain a steady depression of the potential varied between 17 and 38 minutes (Mean  $26 \pm 1.7$ ,  $n=9$  for CHA at 50nM). Washout times were longer, requiring up to 60 minutes for recovery to control levels, with some slices failing to attain complete recovery. To overcome this problem, comparable studies were performed using two separate, but matched, slices from the same area of the hippocampus. The percentage decrease by the analogues was determined in both slices, one in the presence of 10 $\mu$ M nifedipine.

Fig. 2.4. The effect of nifedipine on the inhibitory action of other adenosine analogues.

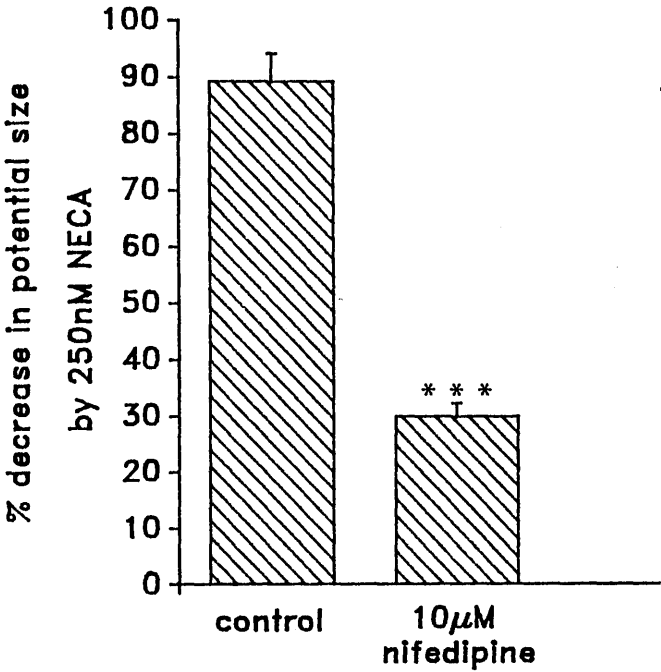


Fig.2.4a. Bars represent the percentage inhibition of the population potential by N-ethylcarboxamidoadenosine (250nM) in the presence or absence of 10µM nifedipine. Mean  $\pm$  sem of 4 determinations. \*\*\*=P<0.01. (PTT).

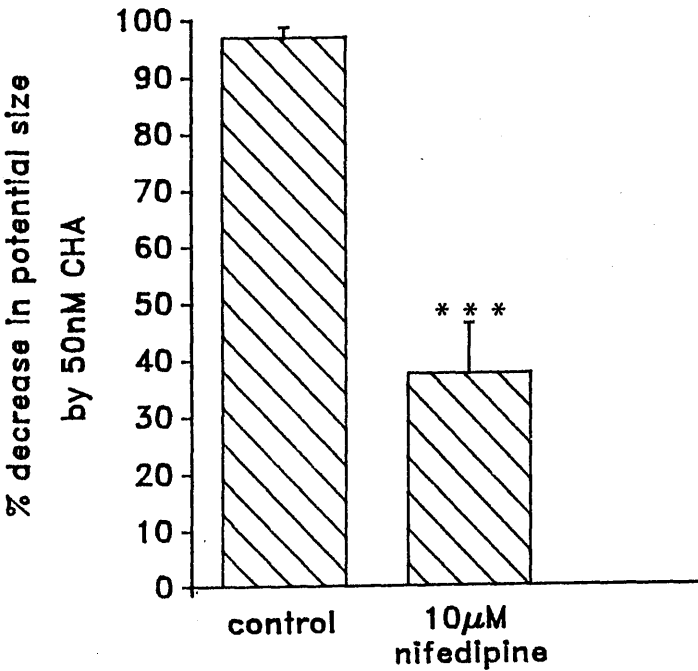


Fig.2.4b. Bars represent the percentage inhibition of the population potential by cyclohexyladenosine (50nM) in the presence or absence of 10µM nifedipine. Mean  $\pm$  sem of 6 determinations. \*\*\* = P<0.001 (PTT).

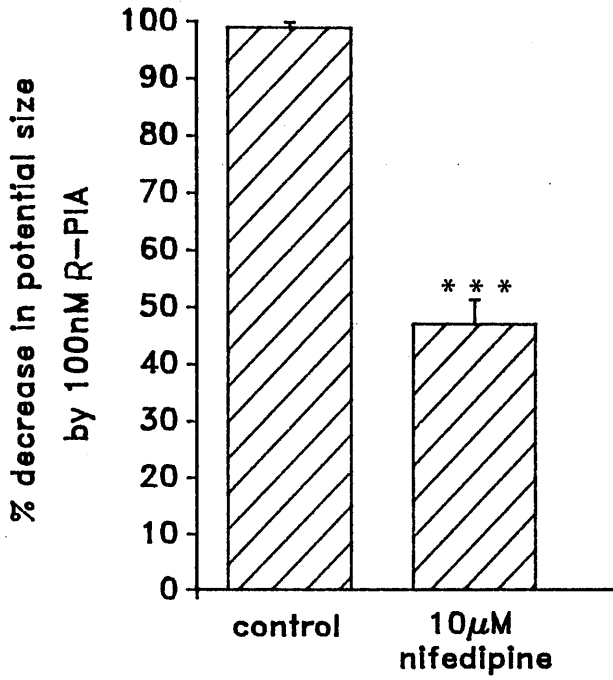


Fig.2.4c. Bars represent the percentage inhibition of the population potential by R-phenylisopropyladenosine (100nM) in the presence or absence of 10µM nifedipine. Mean  $\pm$  sem of 6 determinations. \*\*\* =  $P < 0.001$  (PTT)

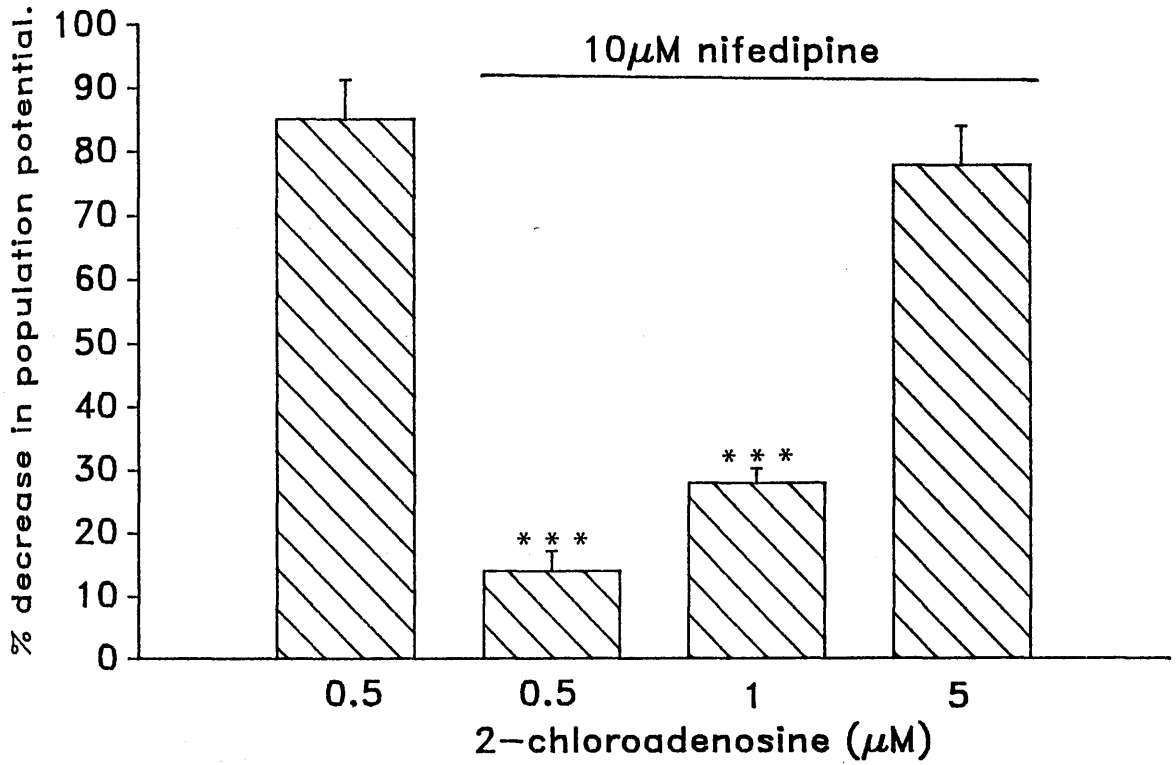
2.3.3. The effect of increasing concentrations of 2-chloroadenosine on the blocking action of nifedipine.

To determine whether the loss of 2-chloroadenosine inhibition was due to a down regulation of adenosine receptors or irreversible binding of nifedipine, increasing concentrations of 2-chloroadenosine were added in the presence of nifedipine (Fig.2.5.).

The antagonism by nifedipine of the inhibitory action of 2-chloroadenosine could be overcome by increasing the concentration of 2-chloroadenosine. Concentrations of 5 $\mu$ M or greater were required to obtain levels of inhibition comparable to control.

After nifedipine perfusion had been discontinued, the percentage decrease of the potential by 2-chloroadenosine did not recover to control levels even after 2 hours perfusion with control ACSF. However, if increasing concentrations of 2-chloroadenosine were added in the post-nifedipine period, the potential could be abolished at concentrations of 5 $\mu$ M and above. Once the 2-chloroadenosine had been washed out pre-nifedipine responses to 2-chloroadenosine (0.5 $\mu$ M) were restored.

Fig. 2.5. The action of increasing concentrations of 2-chloroadenosine on the population potential in the presence of nifedipine.



Bars represent the percentage decrease in the population potential by 2-chloroadenosine. Mean  $\pm$  sem of 5 determinations. \*\*\* =  $P < 0.001$  (PTT).



2.3.4. The action of nifedipine in the presence of adenosine and 2-chloroadenosine.

Experiments were carried out to determine whether nifedipine could exert an effect when adenosine and 2-chloroadenosine were already present.

The addition of 25 $\mu$ M adenosine produced a  $35 \pm 4.3\%$  (n=5) decrease of the population potential. When 10 $\mu$ M nifedipine was added in the presence of adenosine, the potential was gradually reduced by  $75 \pm 4.9\%$  (n=5) over a period of  $28 \pm 1.9$  minutes (n=5).

2-chloroadenosine (0.5 $\mu$ M) inhibited the population potential by  $63 \pm 2.3$  (n=5). The addition of nifedipine (10 $\mu$ M) with 2-chloroadenosine resulted in a gradual recovery of the potential to control levels over 37-50 minutes ( $43 \pm 2.8$ , n=5).

Figures 2.6a & b show the change in the population potential size over time for a single, representative experiment.

Fig. 2.6. Change in the inhibitory action of adenosine and 2-chloroadenosine upon addition of nifedipine.

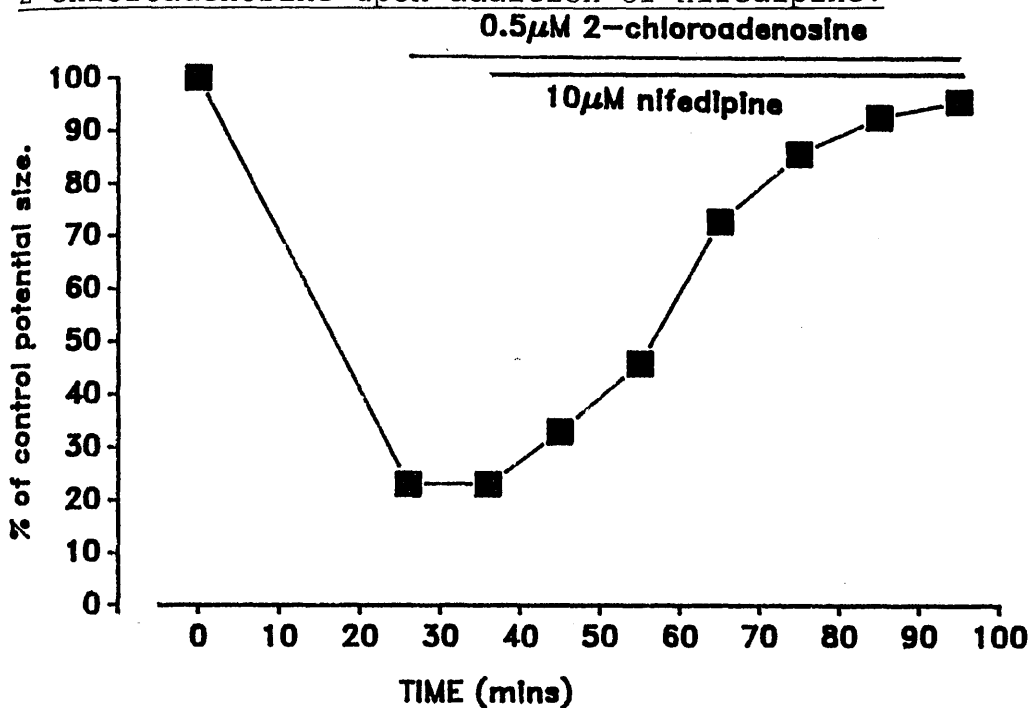


Fig 2.6a. The change in the size of the CA1 evoked population potential over time with 2-chloroadenosine and nifedipine. Points represent the size of the potential as a percentage of the control potential size (100%). Values are taken from one representative experiment only.

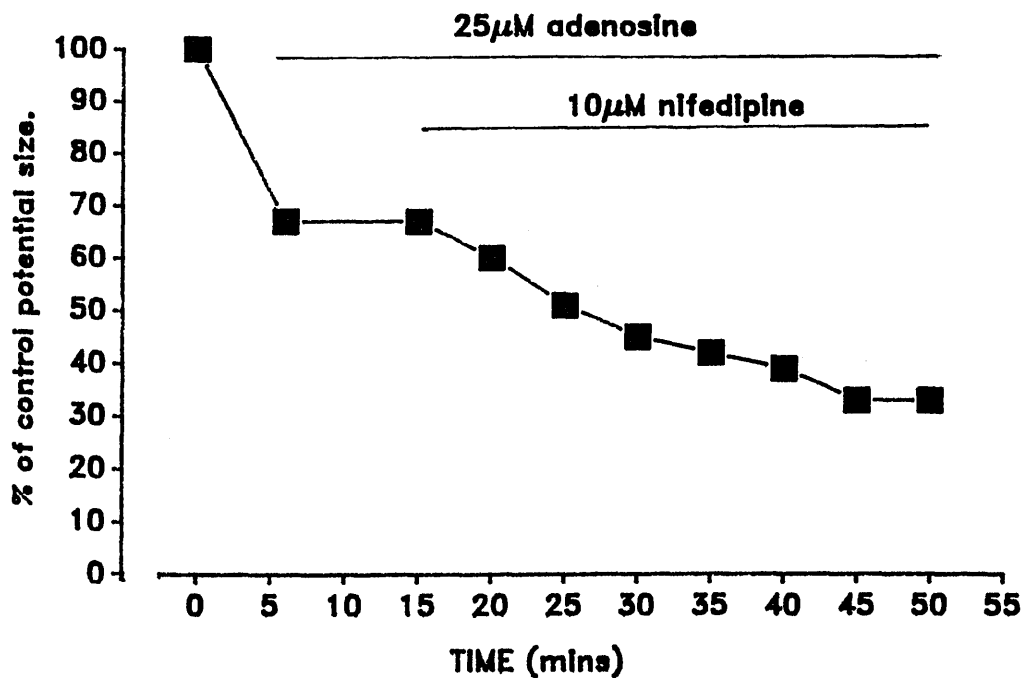


Fig 2.6b. The change in the size of the CA1 evoked population potential over time with adenosine and nifedipine. Points represent the size of the potential as a percentage of the control potential size (100%). Values are taken from one representative experiment only.

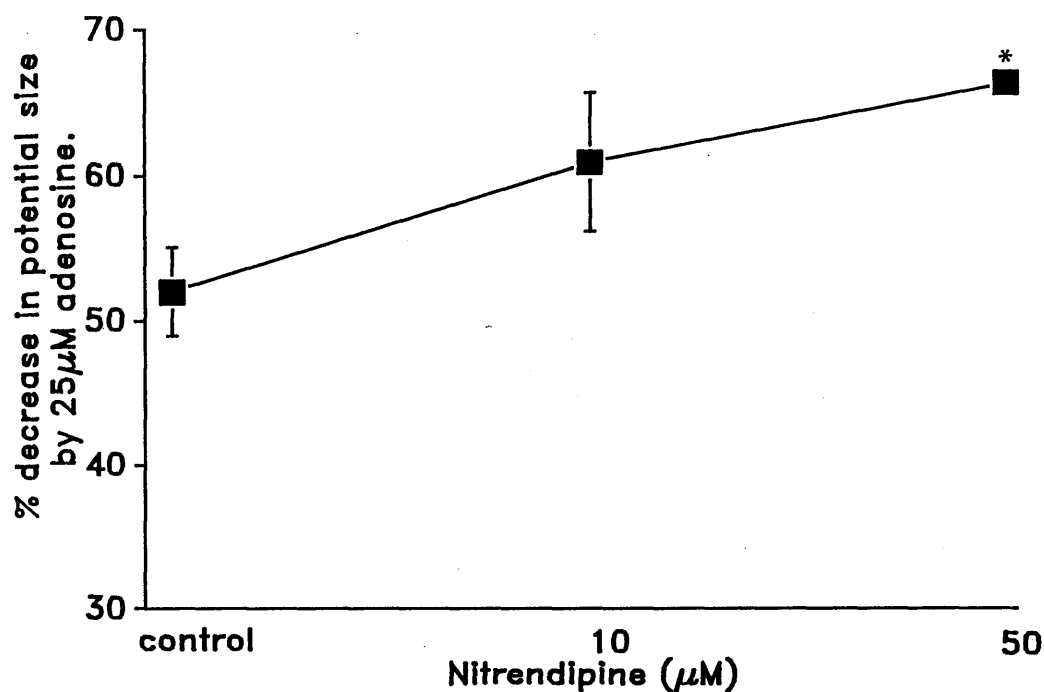
### 2.3.5. The effect of other calcium channel antagonists on adenosine and 2-chloroadenosine.

The effect of the dihydropyridines, nimodipine and nitrendipine and the phenylalkylamine, verapamil on the inhibitory action of adenosine and 2-chloroadenosine was determined.

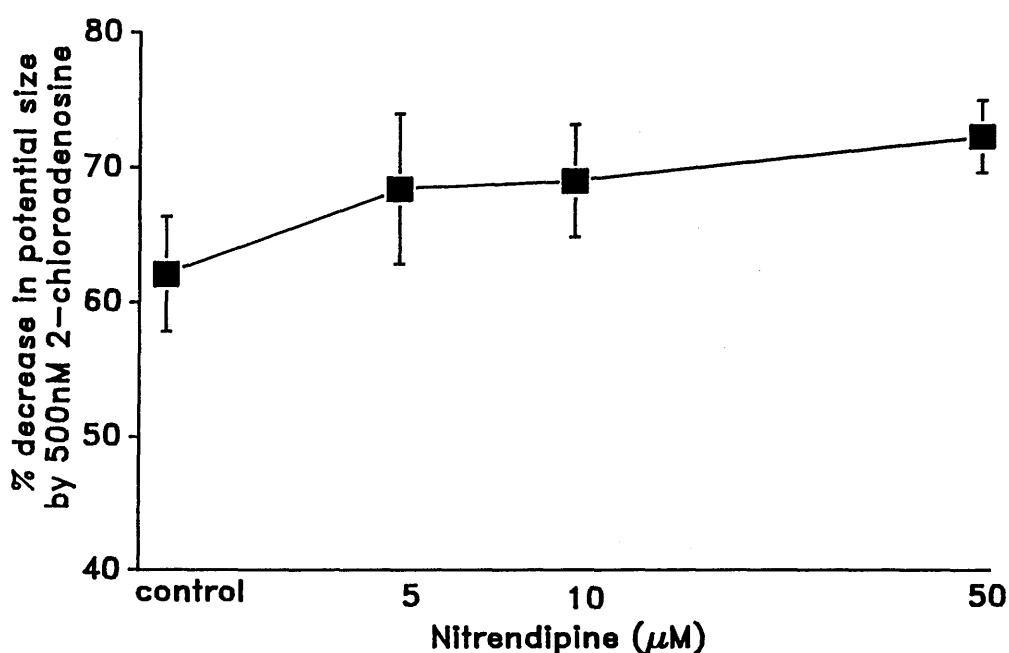
These compounds had a largely non-significant effect on the action of adenosine or 2-chloroadenosine, although there was a small but significant increase in the level of inhibition by both adenosine and 2-chloroadenosine in some experiments.

Verapamil itself depressed the population potential when added at concentrations of 20 $\mu$ M and above, so any increase in inhibition by adenosine in the presence of verapamil cannot be attributed to an interaction between the two compounds.

Fig. 2.7 The effect of nitrendipine on the inhibition of the CA1 population potential by adenosine and 2-chloroadenosine.

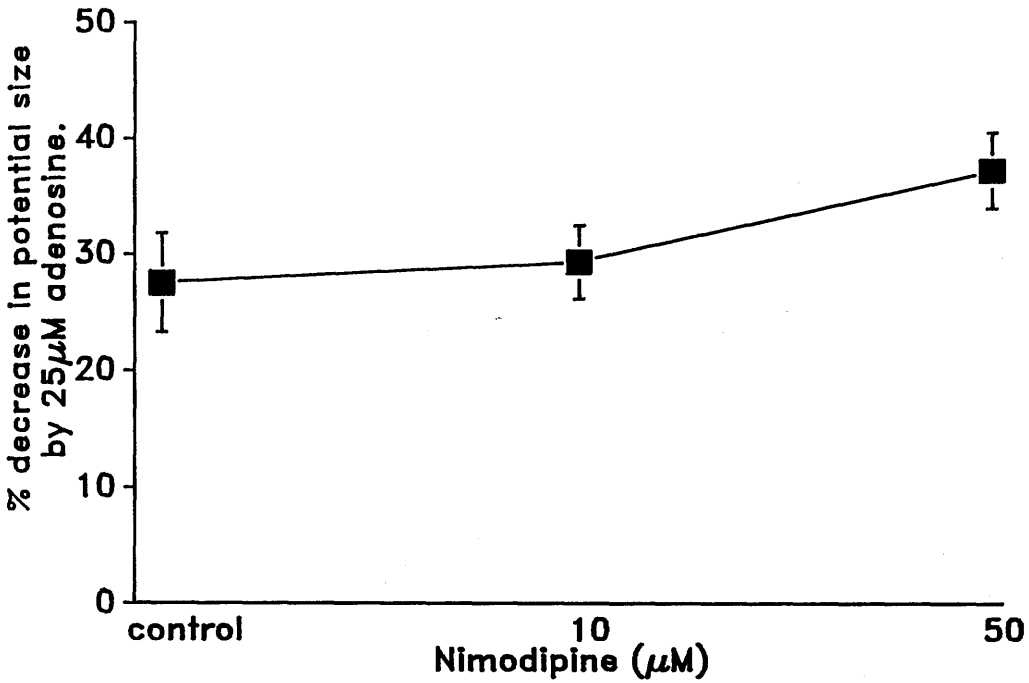


Figs 2.7a. Effect of nitrendipine on the percentage decrease of the population potential by 25  $\mu\text{M}$  adenosine. Points are mean  $\pm$  sem of 4 determinations. \* =  $P < 0.1$  (PTT)

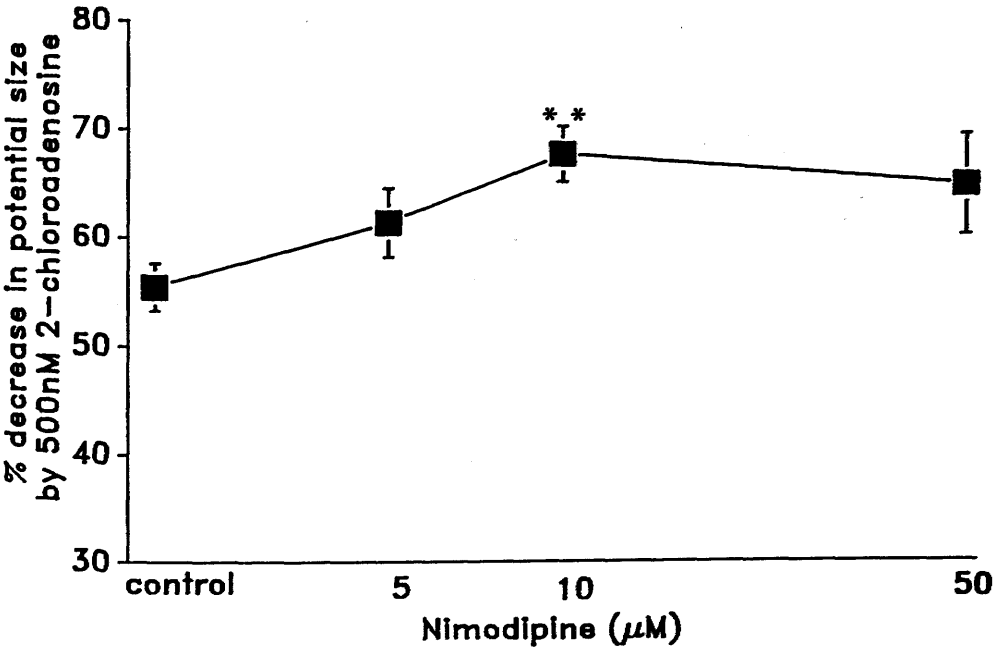


Figs 2.7b. Effect of nitrendipine on the percentage decrease of the population potential by 0.5  $\mu\text{M}$  2-chloroadenosine. Points are mean  $\pm$  sem of 4 determinations.

Fig. 2.8. The effect of nimodipine on the inhibition of the CA1 population potential by adenosine and 2-chloroadenosine.

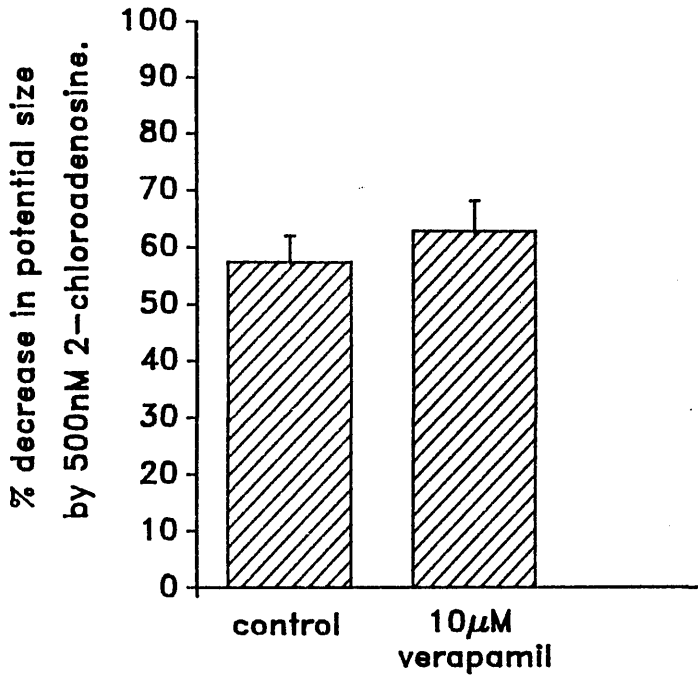


Figs 2.8a. Effect of nimodipine on the percentage decrease of the population potential by 25  $\mu\text{M}$  adenosine. Points are mean  $\pm$  sem of 4 determinations.



Figs 2.8b. Effect of nimodipine on the percentage decrease of the population potential by 0.5  $\mu\text{M}$  2-chloroadenosine. Points are mean  $\pm$  sem of 4 determinations. \*\* =  $P < 0.05$  (PTT).

Fig 2.9. The effect of verapamil on the inhibition of the population potential by 2-chloroadenosine.



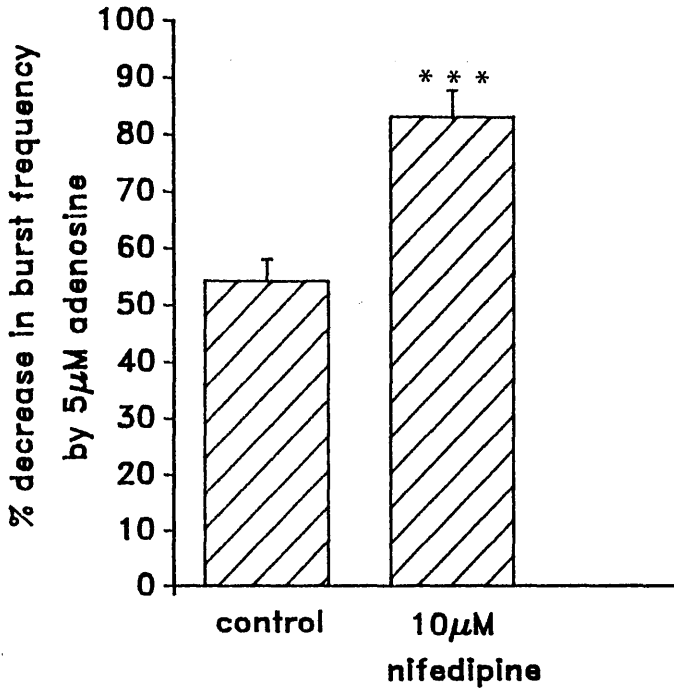
The effect of verapamil on the percentage decrease of the CA1 evoked population potential by 0.5µM 2-chloroadenosine. Bars represent the mean  $\pm$  sem of 5 determinations.

### 2.3.6 The action of nifedipine on the anticonvulsant properties of adenosine and 2-chloroadenosine.

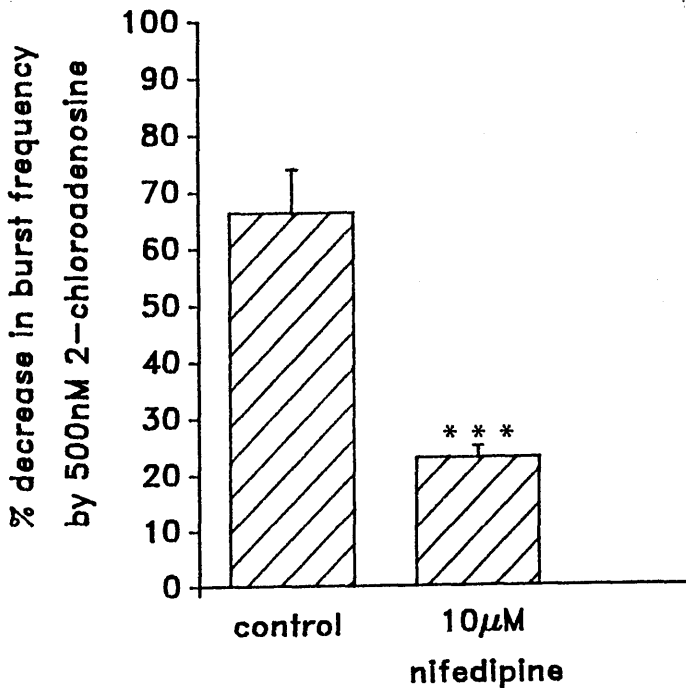
The original experiments showed that nifedipine had no effect on spontaneous activity induced by magnesium free ACSF in the CA3 region of the hippocampal slice. Adenosine, on the other hand, is a potent anticonvulsant in this model of epilepsy. The effect of adenosine and 2-chloroadenosine on spontaneous activity was determined in the presence of nifedipine for comparison with the above studies.

Control responses to adenosine or 2-chloroadenosine were determined in triplicate to ensure the percentage depression of burst frequency remained constant. Nifedipine was then added for 10 minutes and the adenosine responses repeated.

Fig.2.10. The effect of nifedipine on the inhibition of magnesium free ACSF induced CA3 spontaneous activity by adenosine and 2-chloroadenosine.



Figs 2.10a. Effect of nifedipine on the percentage decrease of magnesium free induced bursts by 5μM adenosine. Burst frequency was recorded over 10 minutes. Bars represent the mean  $\pm$  sem of 5 determinations. \*\*\* =  $P < 0.01$  (PTT).



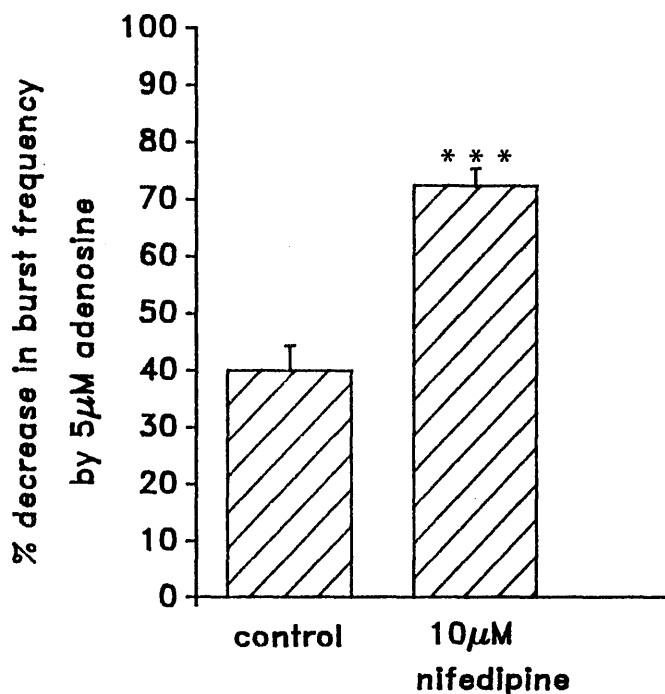
Figs 2.10b. Effect of nifedipine on the percentage decrease of magnesium free induced bursts by 0.5μM 2-chloroadenosine. Burst frequency was recorded over 10 minutes. Points are mean  $\pm$  sem of 5 determinations. \*\*\* =  $P < 0.001$  (PTT).



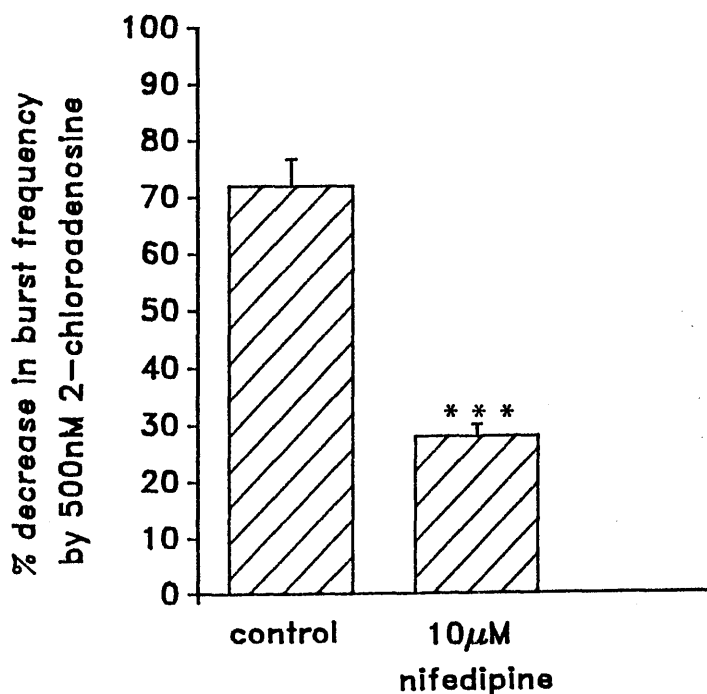
For a comparison with the magnesium free bursts, and to ensure that the observed effect of nifedipine was not due to the absence of magnesium, the experiments were repeated using raised extracellular potassium (8.5mM) to induce spontaneous activity. For these experiments a stable decrease in burst frequency was established with adenosine or 2-chloroadenosine. Nifedipine (10 $\mu$ M) was then added with the adenosine compounds still present (Fig.2.11).

Nifedipine itself had no significant effect on the bursts induced by magnesium free ACSF or high potassium.

Fig.2.11. The effect of nifedipine on the inhibition of 8.5mM potassium induced CA3 spontaneous activity by adenosine and 2-chloroadenosine.



Figs 2.11a. Effect of nifedipine on the percentage decrease of 8.5mM potassium induced bursts by 5μM adenosine. Burst frequency was recorded over 10 minutes. Bars represent the mean  $\pm$  sem of 5 determinations. \*\*\* =  $P < 0.01$  (PTT).



Figs 2.11b. Effect of nifedipine on the percentage decrease of 8.5mM potassium induced bursts by 0.5μM 2-chloroadenosine. Burst frequency was recorded over 10 minutes. Points are mean  $\pm$  sem of 5 determinations. \*\*\* =  $P < 0.001$ . (PTT).

Similar experiments were carried out to determine the effect of verapamil on the action of adenosine on magnesium free ACSF induced bursts.

As verapamil itself inhibited the spontaneous activity (Fig 2.12c.), the apparent increase in the percentage inhibition in the presence of 25 $\mu$ M verapamil was probably due to the overall reduction in burst frequency, rather than any direct effect on the action of adenosine or 2-chloroadenosine by verapamil.

Fig. 2.12 The effect of verapamil on inhibition of magnesium free ACSF induced spontaneous activity by adenosine and 2-chloroadenosine.

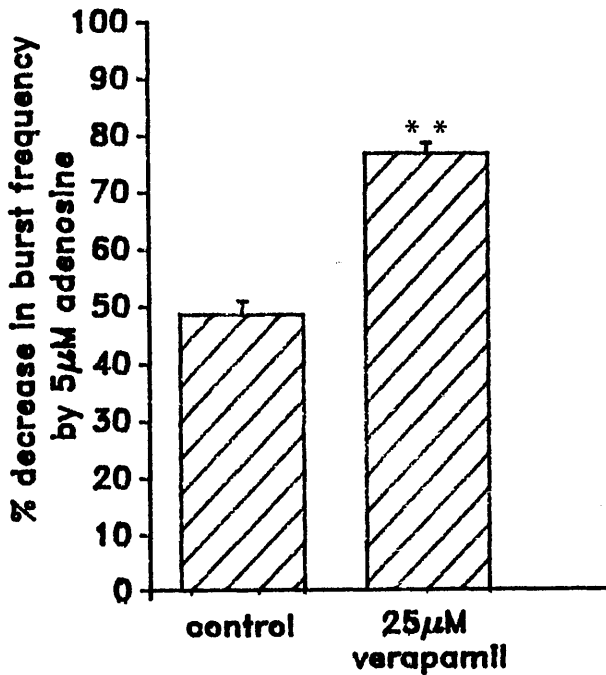
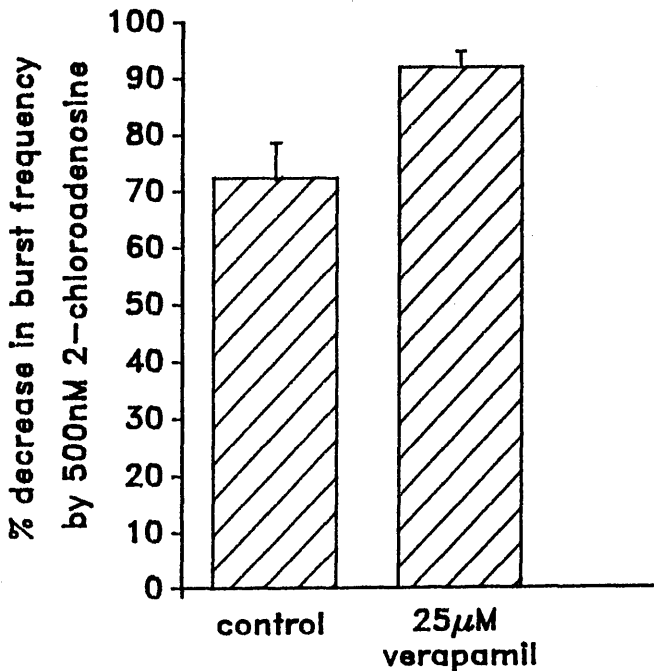


Fig. 2.12a. Effect of verapamil on the percentage decrease of magnesium free induced bursts by 5μM adenosine. Bars represents the mean  $\pm$  sem of 3 determinations. \*\* =  $P < 0.05$  (PTT).



Figs 2.12b. Effect of verapamil on the percentage decrease of magnesium free induced bursts by 0.5μM 2-chloroadenosine. Bars represent the mean  $\pm$  sem of 3 determinations.

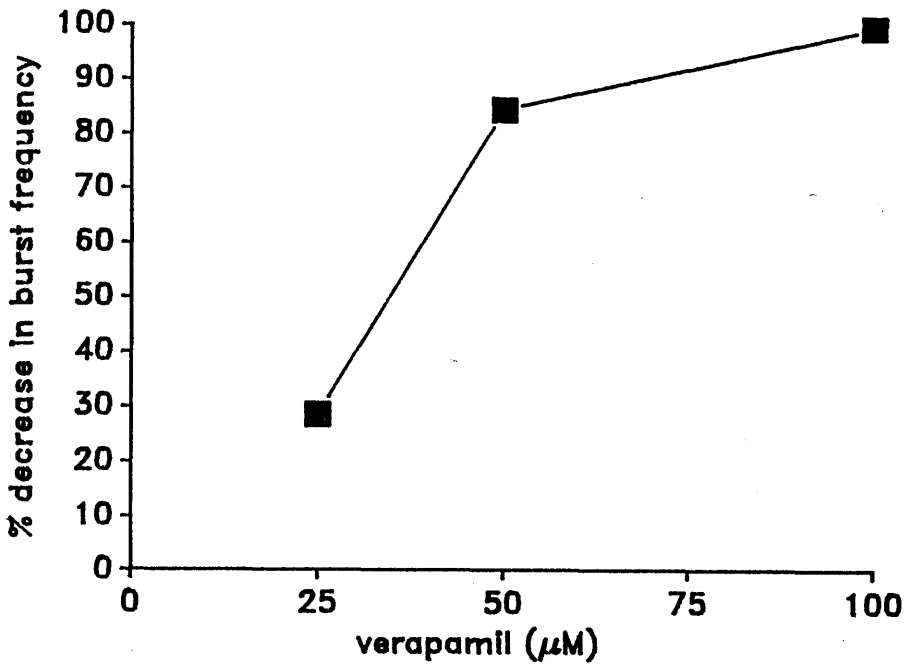


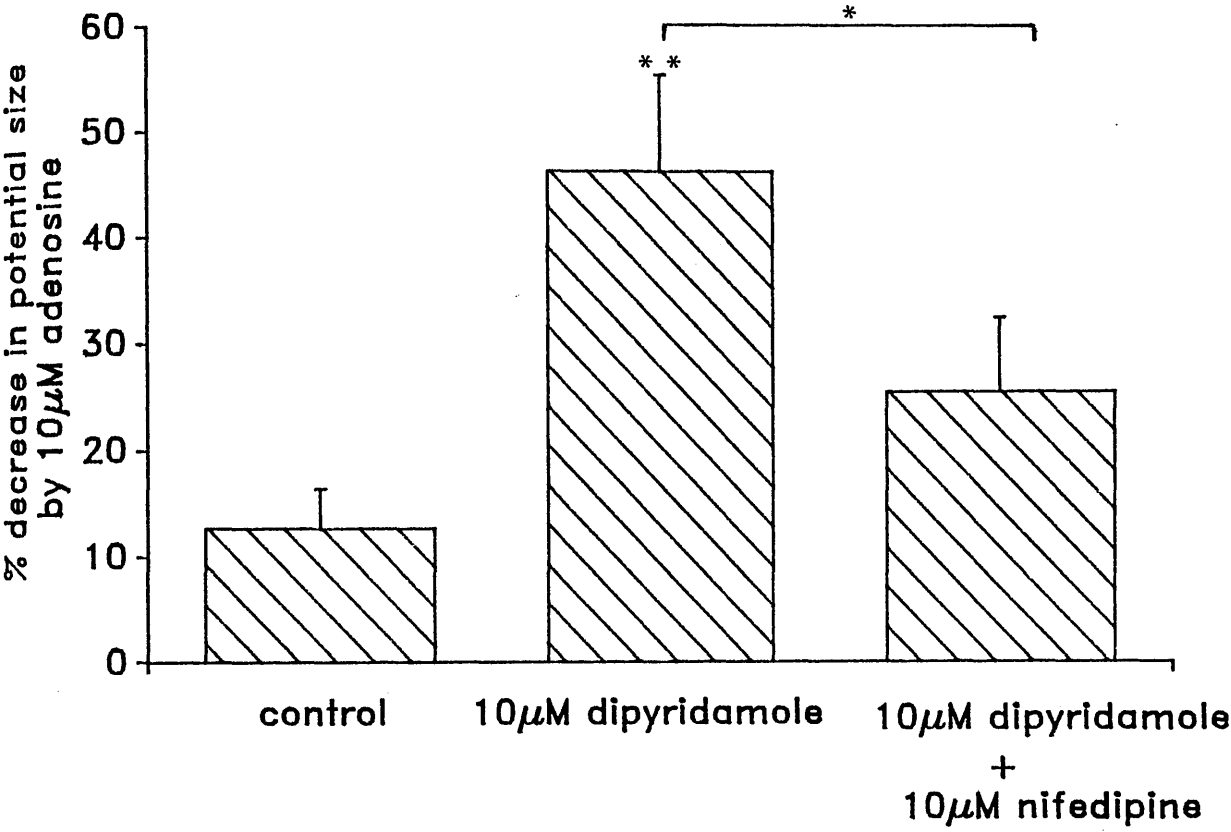
Fig. 2.12c. The effect of verapamil on magnesium free ACSF induced bursts. Points are mean  $\pm$  sem of 3 determinations.

2.3.7. The role of adenosine uptake in increasing the level of adenosine inhibition in the presence of nifedipine.

The key observation of these experiments was the difference between the effect of nifedipine and BayK 8644 on adenosine as compared to its analogues, the effect of adenosine being potentiated while the effect of the analogues was attenuated.

The contribution of the adenosine uptake system to the effect of nifedipine on adenosine was determined. A low concentration of adenosine (10 $\mu$ M) was added and a stable inhibition of the evoked CA1 population potential was obtained. Dipyridamole (10 $\mu$ M), an adenosine uptake blocker, was then added in the presence of the adenosine. This resulted in a significant increase in the level of inhibition by adenosine. The addition of dipyridamole to a slice on its own also resulted in a depression of the potential, presumably as a result of blocking endogenous adenosine uptake. Nifedipine (10 $\mu$ M) was then added to the adenosine and dipyridamole. The result was a significant decrease in the level of inhibition but not to pre-dipyridamole levels.

Fig. 2.13. The effect of nifedipine on adenosine in the presence of an adenosine uptake blocker.



Bars represent the percentage decrease of the CA1 evoked population potential by 10μM adenosine in the presence of dipyridamole and dipyridamole plus nifedipine. Values are mean  $\pm$  sem of 6 determinations. \* =  $P < 0.1$ , \*\* =  $P < 0.05$  (PTT). Inhibition by dipyridamole plus nifedipine is not significantly different from control.

#### 2.4. DISCUSSION

It is apparent from the results reported in this chapter that the dihydropyridines have little effect on evoked or spontaneous neuronal activity in the hippocampal slice. In magnesium free ACSF, the principle initiator of spontaneous activity is an influx of calcium across voltage dependent and/or NMDA operated calcium channels. If the dihydropyridines have any effect on these channels then a change in the burst frequency would be expected to occur. Some studies have reported that a small proportion of calcium channels in neuronal tissue may be affected by the dihydropyridines (Docherty and Brown, 1986), but these appear to play no part in the activation of spontaneous activity or evoked synaptic transmission. Other reports of the action of the dihydropyridines on epileptiform models are limited and largely negative. Frank et al.(1988) showed that epileptiform bursts induced in the rat hippocampal slice by caffeine, 4-aminopyridine and penicillin were all unaffected by nifedipine at concentrations up to 100 $\mu$ M.

Studies of the dihydropyridines on epileptic seizures in vivo have shown a varied action. Vezzani et al.(1988), studying limbic seizures in rats induced by injections of kainic or quinolinic acid into the hippocampus, found that nifedipine injected intraperitoneally at 40mg/kg was ineffective against the seizures. However, nifedipine was effective against kindled seizures, blocking them completely at 40mg/kg. In similar in vivo studies in rabbits,



systemically administered nimodipine was effective against seizures induced by electrical stimulation of the cortical surface (Meyer et al.,1986a), seizures induced by cefazolin (Morocutti et al.,1988) and seizures induced by ischemia, bicuculline and pentylenetetrazol (Meyer et al.,1986b). Thus, it appears that the dihydropyridines are able to prevent or inhibit seizures in some animal models of epilepsy. However, as all the in vivo models involved peripheral administration of the dihydropyridines, it is possible that peripheral actions of the dihydropyridines, such as an increase in cerebral blood flow, were responsible for the attenuation of the bursts. In the study by Vezzani et al.(1988), the use of kainic or quinolinic acid produced bursts that were unaffected by nifedipine. Quinolinic acid activates NMDA receptors to produce seizures, as is the case with magnesium free ACSF, so nifedipine appears to have no effect on excitatory amino acid mediated epileptiform activity.

However, when the action of the calcium channel blockers on evoked or spontaneous activity is studied in conjunction with adenosine, there is a clear interaction between the inhibitory action of adenosine and its analogues, and the dihydropyridine class of calcium antagonists. This appears to involve more than one type of interaction, as the effects of the dihydropyridines on adenosine is the opposite of that observed with the adenosine analogues. The dihydropyridines are known to bind to distinct, high affinity binding sites

in brain tissue (Belleman et al., 1983) which may be associated with calcium channels (Skattebol and Triggle, 1987). However, it does not appear that the dihydropyridines are exerting an effect on adenosine via a direct action on calcium channels as both the dihydropyridine channel antagonist (nifedipine) and agonist (BayK 8644) exert the same effect on adenosine. Also, verapamil, that has a weak effect on neuronal calcium channels, but stronger than the dihydropyridines (Wei and Chang, 1986; White and Bradford, 1986), had no effect on adenosine. As adenosine is thought to act via calcium channels, the possibility arises that receptors for adenosine and the dihydropyridines may occur in close proximity, possibly associated with the same calcium channel.

The action of the dihydropyridines on adenosine was to increase the level of inhibition by adenosine. It is possible that a small decrease in calcium influx by the dihydropyridines, acting as calcium channel blockers, is being enhanced by adenosine, resulting in an apparent increased inhibition by adenosine. The dihydropyridines on their own do not decrease the evoked potential, but the action of adenosine may be to enhance the weak effect of the dihydropyridines and increase the total inhibition on calcium channels. However, this does not seem likely as both the calcium channel antagonist and the agonist BayK 8644 had the same effect on adenosine. Also, the effect of the dihydropyridines on the adenosine analogues is to reduce

their inhibitory action, an effect that cannot readily be explained by a decreased calcium influx. The evidence for an action of dihydropyridines on neuronal calcium channels is also very poor. Thus, an inhibition of calcium channels by the dihydropyridines, to explain the increased inhibition by adenosine, can effectively be ruled out.

Another possibility that needs to be considered is that the increase in the inhibition by adenosine may be due to a simple decrease in the viability of the slice, which does occur after a number of hours (>6) at 30°C. However, this can be ruled out for a number of reasons. The increased inhibition by adenosine occurred immediately after a 20 minute period of perfusion with nifedipine or BayK 8644, too rapid to be due to slice deterioration. There was no decrease in the normal potential size, which is usually observed in deteriorating slices. The ethanol vehicle had no effect on adenosine, even at 1%, a concentration higher than that used for vehicle (0.1%). A series of experiments were carried out where nifedipine was added in the presence of adenosine (Fig.2.6a). The presence of nifedipine gradually increased the inhibition of the population potential by adenosine. This indicates that the enhanced effect of adenosine is due to the nifedipine and not to any decrease in slice viability.

The effect of the dihydropyridines on the adenosine analogues was to attenuate the inhibition of the population potential by the analogues. This response does not suggest an inhibition of calcium influx, although a number of other possible effects can be considered.

A simple chemical interaction in the ACSF was considered. It is always possible that when two compounds are mixed in solution, one may affect the other so as to impair its activity. This possibility was eliminated by adding ACSF containing 2-chloroadenosine and nifedipine direct to the perfusion system. The potential was decreased by the same percentage as with 2-chloroadenosine alone. This indicates that; 1) there is no inactivation of the 2-chloroadenosine by nifedipine, 2) the effect of nifedipine is not an immediate interaction, but requires time to bind to its own receptor or displace 2-chloroadenosine from the adenosine receptor.

It is also possible that there is an apparent desensitization per se of the adenosine receptors to explain the loss of response to the adenosine analogues. This seems unlikely as consistent responses to 2-chloroadenosine and NECA were performed in triplicate before the addition of nifedipine and BayK 8644. If a high concentration of the dihydropyridines (10 $\mu$ M) was then added and perfused for 10 minutes or more, the response to subsequent additions of the analogues was abolished. Similarly, if 2-chloroadenosine was added and maintained for up to 30 minutes after a maximal

inhibition had been achieved, there was no reduction in the percentage inhibition. If, however, nifedipine (10 $\mu$ M) was added while the 2-chloroadenosine was still present, the inhibition by 2-chloroadenosine could be totally abolished after 37-50 minutes ( $43 \pm 2.8$  mins,  $n=5$ ) (Fig.2.6b). This demonstrates that the decreased efficacy of 2-chloroadenosine is a result of the presence of the nifedipine, as opposed to any non-specific "desensitization" of adenosine receptors.

The most plausible explanation for the effect of the dihydropyridines on adenosine and the adenosine analogues can be obtained from studies of the interactions between calcium channel antagonists and adenosine compounds.

Murphy and Snyder (1983) were the first to report the displacement of [ $^3$ H]CHA binding by nifedipine. They used rat and bovine brain membranes to study the effect of a range of calcium channel blockers on [ $^3$ H]CHA and [ $^3$ H]DPX (1,3-diethyl-8-phenylxanthine) binding. Verapamil and diltiazem had no effect on [ $^3$ H]CHA or [ $^3$ H]DPX binding, while nimodipine and nitrendipine displaced [ $^3$ H]CHA binding at relatively high concentrations only ( $IC_{50}$ : nimodipine =  $>100\mu$ M, nitrendipine =  $60\mu$ M). However, nifedipine potently displaced both [ $^3$ H]CHA and [ $^3$ H]DPX with an  $IC_{50}$  of 4 and  $0.5\mu$ M respectively. Studies of [ $^3$ H]nitrendipine binding in both rat heart and brain showed that the other dihydropyridines could displace [ $^3$ H]nitrendipine, but verapamil and diltiazem were

ineffective. This indicates that the dihydropyridines bind to a distinct receptor site separate from that at which verapamil and diltiazem act. Taking the study one step further, Murphy and Snyder determined the  $B_{max}$  and Hill coefficient of the effect of nifedipine on [ $^3H$ ]CHA binding. These values did not correlate with a simple competitive inhibition and suggested that the dihydropyridines and adenosine analogues bind to separate sites, the binding of the dihydropyridines possibly allosterically modulating adenosine binding.

A study by Phillis et al.(1984) demonstrated functional interactions between the dihydropyridines and adenosine by showing the ability of nifedipine to attenuate [ $^3H$ ]adenosine uptake into rat cortical synaptosomes. This occurred in a dose dependent manner with an  $IC_{50}$  of 1.1 $\mu$ M for nifedipine. In the same study the inhibitory action of iontophoretically applied adenosine on the acetylcholine stimulated firing of single cortical neurones of the rat in vivo, was shown to be markedly potentiated by the addition of nifedipine. This could be explained by the inhibition of adenosine uptake by nifedipine increasing the level of endogenous adenosine.

In a later study by Fredholm et al.(1986), the effect of BayK 8644 and nifedipine on [ $^3H$ ]noradrenaline release from hippocampal slices was studied. BayK 8644 and nifedipine had no effect on electrically stimulated [ $^3H$ ]noradrenaline release on their own, while R-PIA inhibited release by 25%.

However, in the presence of BayK 8644 (1 $\mu$ M), R-PIA inhibition of release was completely blocked. This was not the case with nifedipine which had no effect on R-PIA at 1 $\mu$ M. Ligand binding studies performed by the same group showed both BayK 8644 and nifedipine to displace [ $^3$ H]R-PIA binding. However, the displacement by BayK 8644 at 1 $\mu$ M was comparatively weak, reducing R-PIA binding by only 15% at sub-optimal concentrations of R-PIA, as compared to the effect of 1 $\mu$ M BayK 8644 on [ $^3$ H]noradrenaline, where release was completely blocked. This suggests that BayK 8644 is not simply displacing R-PIA to reduce its effect. These studies were subsequently extended (Hu et al., 1987) to show that the inhibition of R-PIA action by BayK 8644 had no effect on the ability of R-PIA to reduce cAMP accumulation. Similarly, there was no effect of added GTP on the interaction of BayK 8644 and R-PIA binding, nor was there any difference between binding in intact and solubilized membranes. This suggests that BayK 8644 is not acting via any interaction with the G-proteins that are thought to link the adenosine receptor with its effector site. The fact that verapamil and diltiazem do not share this interaction with adenosine analogues was also confirmed.

Other, more extensive, ligand binding studies have also reported the ability of the dihydropyridines to interact with adenosine receptors. Morgan et al. (1987) showed that binding to  $\alpha$  and  $\beta$  adrenergic, muscarinic, opiate, GABA and benzodiazepine receptors by their respective ligands are all

unaffected by nifedipine in rat cortical membranes, while both [ $^3$ H]CHA and [ $^3$ H]DPX binding was potently inhibited by nifedipine and BayK 8644. Nitrendipine and nimodipine displaced [ $^3$ H]DPX, but were ineffective against [ $^3$ H]CHA, a result similar to that reported by Murphy and Snyder (1983). The binding of [ $^3$ H]NBI, a ligand specific to the nucleoside transport (adenosine uptake) system, was shown to be reduced by the dihydropyridines in a competitive manner, BayK 8644 and nifedipine being equipotent, with nitrendipine and nimodipine slightly less effective. Again verapamil and diltiazem were ineffective. They also repeated the work of Phillis et al. (1984) and showed that uptake of [ $^3$ H]adenosine into synaptosomes was significantly inhibited by the dihydropyridines, with nitrendipine and nimodipine being slightly more potent than nifedipine. This demonstrates that the dihydropyridines can also affect the adenosine uptake system. Verapamil had no effect on uptake, although diltiazem was effective at inhibiting uptake.

A final experiment by Morgan et al. (1987) was to look at the effect of the adenosine analogues, CHA and 2-chloroadenosine, on [ $^3$ H]nitrendipine binding. This showed there was no effect in the reciprocal situation,  $K_i$  being greater than 200  $\mu$ M in both cases, suggesting that the dihydropyridines are acting on adenosine binding sites, as opposed to any shared adenosine / dihydropyridine receptor site. A similar study was carried out by Cheung et al. (1987) with comparable results.



All these studies show that the dihydropyridines, in particular nifedipine and BayK 8644, can affect the binding of adenosine analogues to the adenosine receptor (probably the A<sub>1</sub> receptor) and can also interact with the adenosine uptake system to promote [<sup>3</sup>H]adenosine uptake or displace [<sup>3</sup>H]NBI. From these studies it is possible to explain the results reported in this chapter.

The dihydropyridines appear to bind to a distinct site that interacts with, but is not the same receptor as, the adenosine receptor. Analysis of ligand binding indicates there is not a straightforward competitive inhibition between nifedipine and adenosine analogues (Murphy and Snyder, 1983), although scatchard analysis by Morgan et al.(1987) suggests a mixed competitive / non-competitive inhibition, with both the K<sub>D</sub> and B<sub>max</sub> altering, indicating a decrease in the number of adenosine binding sites and the receptor affinity. This suggests the dihydropyridines are exerting an allosteric modulation of the adenosine receptor via a receptor site, the identity of which remains unclear. As mentioned earlier, ligand binding studies suggest that the effect of the dihydropyridines does not appear to be exerted via the dihydropyridine calcium channel receptor, neither via action on a calcium channel nor, according to Morgan et al.(1987), via allosteric modulation of the adenosine receptor. This last conclusion is drawn because of the differential effect of the different dihydropyridines, nifedipine and BayK 8644 being very effective, nimodipine

and nitrendipine virtually ineffective, while all these ligands can bind with high affinity to brain dihydropyridine receptors (Belleman et al., 1983). Also, the concentrations of the dihydropyridines required to exert an effect on adenosine are very much higher than those required to exert a maximal effect in smooth muscle.

However, it is always possible that the dihydropyridines can bind to a low affinity site on the same receptor molecule once the high affinity sites are fully saturated. All the dihydropyridines studied could displace the adenosine antagonist [ $^3\text{H}$ ]DPX, which presumably binds to the adenosine receptor, so an allosteric interaction direct from the main dihydropyridine receptor cannot be ruled out.

Another possibility is the existence of a distinct low affinity dihydropyridine receptor site, specific for nifedipine and BayK 8644, which allosterically modulates adenosine binding sites. This may exist on the same receptor molecule as the high affinity binding sites as suggested above, or it may be a separate receptor located near the adenosine receptor. The existence of distinct high and low affinity binding sites for BayK 8644 have been demonstrated in cardiac tissue (Sarmiento et al., 1987). The high affinity site is thought to be associated with calcium channels while the function of the low affinity site is unclear. If a similar situation exists in neuronal tissue, then the low affinity site may be involved in modification of the adenosine binding site. There is a structural difference

between the active and inactive dihydropyridines that could allow for a specific receptor site for subclasses of dihydropyridines. Nifedipine and BayK 8644 have a nitro ( $\text{NO}_2$ ) group on site 3 of the phenol ring, as does nisoldipine which is also an active displacer of [ $^3\text{H}$ ]CHA ( $\text{IC}_{50}=15\mu\text{M}$ ) (Murphy and Snyder, 1983), while nitrendipine and nimodipine are 2-nitrophenol derivatives (Fig.3).

If an allosteric interaction is taking place, this would presumably act by decreasing the number of binding sites, as indicated by ligand binding studies. However, an argument against a specific allosteric down regulation of the number of adenosine binding sites can be obtained from an observation in the present studies that has not been reported in other functional studies. The reduction in inhibition by 2-chloroadenosine in the presence of nifedipine could be overcome by increasing the concentration of 2-chloroadenosine. Also, the use of higher concentrations of 2-chloroadenosine during the post-nifedipine phase restored responses to 2-chloroadenosine to control levels. This suggests a straight-forward competitive inhibition with 2-chloroadenosine displacing nifedipine if added in high enough concentrations. Morgan et al.(1987) showed that adenosine analogues could not displace [ $^3\text{H}$ ]nitrendipine binding, suggesting that an increase in 2-chloroadenosine concentration could not simply displace the dihydropyridines from their receptor to relieve the allosteric hinderance. Of course it is possible that a distinct site exists, as

described above, where only nifedipine and BayK 8644 can bind, and where 2-chloroadenosine can interact to displace the dihydropyridines at high concentrations ( $> 1\mu\text{M}$ ). Studies of [ $^3\text{H}$ ]nitrendipine binding would not show this action. A study of the effects of adenosine analogues on the displacement of [ $^3\text{H}$ ]nifedipine and [ $^3\text{H}$ ]BayK 8644 may resolve this argument. Whatever form the interaction takes, it is clear that adenosine analogue binding is hindered in the presence of the dihydropyridines nifedipine and BayK 8644. This will need to be taken into account should any adenosine analogues come into clinical use.

The effect of the dihydropyridines on adenosine is exerted via a different route. This appears to involve an action of the dihydropyridines on the nucleoside transport system, reducing the uptake of adenosine and subsequently enhancing its action by increasing its availability. The action of the dihydropyridines on the nucleoside transport system, as with the adenosine receptors, appears to be differential, with nifedipine and BayK 8644 being more effective than nimodipine and nitrendipine. The effect on adenosine uptake is indicated by an inhibition of [ $^3\text{H}$ ]adenosine uptake into brain synaptosomes (Phillis et al., 1984; Morgan et al., 1987), and by [ $^3\text{H}$ ]NBI binding studies (Marangos et al., 1984; Morgan et al., 1987). The effect of the dihydropyridines on the nucleoside transporter has also been reported in other tissues including human erythrocyte ghost membranes (Striessnig et al., 1985).

Marangos et al.(1984) used dog brain and heart membranes to show that [ $^3\text{H}$ ]NBI binding could be potently inhibited by the dihydropyridines with nimodipine being the most potent (nimodipine,  $K_i=21\text{nM}$ ; nifedipine,  $K_i=4600\text{nM}$  in brain). Verapamil and diltiazem exhibited low potencies only. In this study, Scatchard analysis indicated that inhibition of [ $^3\text{H}$ ]NBI binding by the dihydropyridines was strictly non-competitive, in contrast to the study by Morgan et al.(1987). Other differences are also apparent between the two studies, in particular the relative potency of the different dihydropyridines in displacing [ $^3\text{H}$ ]NBI. Nifedipine is equipotent but nimodipine is of a significantly lower potency in rat brain ( $K_i=16700\text{nM}$  c.f.  $21\text{nM}$ ). This loosely corresponds to the results obtained in the present experiments where nimodipine and nitrendipine had little effect on adenosine inhibition. However, nimodipine and nitrendipine were able to displace [ $^3\text{H}$ ]NBI at relatively low  $K_i$  values (nim= $16.7\mu\text{M}$ , nit= $7.95\mu\text{M}$ ), but this does not correlate with the present functional studies. The reason for this is not clear, but as binding studies do not necessarily dictate the functional profile of a drug, neuronal dihydropyridine studies being a good example, no great emphasis will be placed on this discrepancy.

To show that the dihydropyridines are acting to impair adenosine uptake, experiments were carried out using dipyridamole to block the nucleoside transport system. Dipyridamole ( $10\mu\text{M}$ ) increased the potency of  $10\mu\text{M}$  adenosine

3.6 fold ( $n=5$ ). When nifedipine ( $10\mu\text{M}$ ) was added in the presence of adenosine and dipyridamole, the inhibition by adenosine was reduced over a period of 30 minutes. This appears to support the hypothesis that nifedipine is acting on the nucleoside transport system, as nifedipine is unable to increase the potency of adenosine in the presence of a more effective uptake blocker. In fact, the action of nifedipine on adenosine appears to be reversed.

There may be two possible explanations for this. Nifedipine may be acting to reduce the action of adenosine at its receptor, as occurs with the analogues. The presence of dipyridamole prevents nifedipine from interacting with the nucleoside transport system and allows it to exert a maximal effect on the adenosine receptor. However, the level of inhibition by adenosine does not return to, or fall below, control (pre-dipyridamole) levels in the presence of nifedipine, so there is no evidence that the nifedipine is directly reducing the inhibitory action of adenosine at its receptor.

Alternatively, nifedipine is acting by displacing dipyridamole from the uptake site, allowing more adenosine to be taken up and reducing the inhibition of the population potential. This would suggest that the action of nifedipine on the nucleoside transport system is relatively weak, at least in comparison with dipyridamole. This is supported by the fact that nifedipine does not further enhance the action of adenosine in the presence of dipyridamole. Similarly, if nifedipine was a potent adenosine uptake blocker, it might

be expected to enhance the action of endogenous adenosine when added on its own, as was the case with dipyridamole where there was an 18% inhibition of the population potential, presumably as a result of enhanced inhibition by endogenous adenosine. Thus, the dihydropyridine compounds can interact with the nucleoside transporter to increase the level of extracellular, active adenosine.

One matter that requires consideration is why adenosine and the analogues do not appear to be affected by both actions of the dihydropyridines; inhibition of nucleoside transport and decreased ligand binding.

The adenosine analogues are thought to be poor ligands for the nucleoside transporter (Miller and Paton, 1979), although 2-chloroadenosine in particular is known to bind (Jarvis et al., 1985) and 2-chloroadenosine and CHA can displace [ $^3\text{H}$ ]NBI binding (Marangos et al., 1982). However, despite binding with the transporter binding sites, the analogues do not appear to be transported into the cells (Clanachan et al., 1986). So the overall potency, already greater than adenosine, does not increase significantly if the uptake system is inhibited by the dihydropyridines. What is not clear is, if adenosine receptor binding for the analogues is being impaired by the dihydropyridines, why does this not impair the action of adenosine, counteracting the increased potency of adenosine due to the inhibited adenosine uptake system?. It is possible that the concentration of adenosine used in these experiments (25  $\mu\text{M}$ )

is sufficiently high to overcome any block of adenosine receptors by nifedipine, particularly after its potency has been increased by reduced adenosine uptake. This did not occur with the adenosine analogues as there was no interaction of the analogues with the nucleoside transport system and therefore no increased potency. Higher concentrations of 2-chloroadenosine, however, did overcome the block, supporting the above possibility. Similarly, in experiments where an adenosine uptake blocker was present, lower concentrations of adenosine were used (10 $\mu$ M) and a small decrease in inhibition by adenosine was observed, possibly as a result of nifedipine blocking the adenosine receptors.

A final discrepancy that needs to be discussed is the effect of nifedipine on human patients mentioned in the introduction to this chapter. Patients being treated for angina with nifedipine have been reported to show increased heart problems in a small number of cases which, according to experiments on dog heart (Merrill et al., 1983), may be due to an inhibition of the action of endogenous and added adenosine on vasodilatation. The results reported in this chapter, and the apparent explanation from ligand binding studies, do not appear to explain the interactions in the heart. If nifedipine acts to increase the potency of adenosine by blocking adenosine uptake, then an enhanced vasodilatation would be expected. There may be two possible explanations for this discrepancy. The vasodilatation of



smooth muscle is thought to act via an  $A_2$  receptor (Kusachi et al., 1983). An increase in the potency of endogenous adenosine may act on  $A_1$  receptors to counteract the  $A_2$  vasodilatory effects. However, as  $A_1$  receptors do not cause vasoconstriction and there is no evidence for  $A_1$  receptors directly antagonizing  $A_2$  effects, this possibility seems unlikely. Alternatively, vasodilatation may act via ATP input, while enhanced  $A_1$  activation would exert the opposite effect. Either way, it is apparent that the adenosine system in the heart does not respond in the same way to the dihydropyridines as that observed in the brain.

Referring back to the behavioural effects of the dihydropyridines discussed in the main introduction (Page 38), the majority of the behavioural effects observed; mild sedation, interference with sleep patterns, ataxia that could be overcome under stress and inhibition of seizures, could be explained by an increase in the action of adenosine as a result of inhibition of the nucleoside transporter by the dihydropyridines. The behavioural effects of the dihydropyridines may result, in part, from an action on the adenosine system, rather than any action on calcium channels.

This chapter has demonstrated the effect of calcium channel blockers, principally the dihydropyridines, on the action of adenosine in in vitro electrophysiological responses in the rat hippocampus. This appears to involve an

action of 2-nitro dihydropyridines on both adenosine receptors, competitively blocking their action, and the nucleoside transport system, inhibiting its action and increasing the availability and potency of adenosine. The possible relevance this might have to the role of calcium channels in the action of adenosine will be looked at in the general discussion.

## CHAPTER 3

### CHAPTER 3

## THE ACTION OF ADENOSINE ON SYNAPTOSOMAL VOLTAGE SENSITIVE CALCIUM CHANNELS.

### 3.1. INTRODUCTION

The exact site, or sites, of action of adenosine have not, as yet, been fully elucidated. In the peripheral nervous system the inhibitory action of adenosine is due to a reduction in the release of neurotransmitter from nerve terminals (Ginsborg and Hirst, 1972). In the central nervous system the site of action of adenosine cannot be determined as easily, although adenosine is able to reduce neurotransmitter release from brain slices (Dolphin and Archer, 1983; Corradetti et al., 1984) and synaptosomes (Michaelis et al., 1979), suggesting a presynaptic site of action for adenosine.

A well established prerequisite for neurotransmitter release is calcium influx (Augustine et al., 1987). If adenosine is acting on neurotransmitter release, a possible route of action is on calcium mobilization into or within the synaptic terminal. This may be via a number of routes: (1) By partially inhibiting voltage sensitive calcium channels (Wu et al., 1982). (2) By inhibiting receptor operated calcium channels. (3) By reducing the effects of calcium at intracellular sites (e.g. synaptic vesicles, Silinsky 1986a). (4) By increasing calcium sequestration by intracellular organelles (Dutta et al., 1984). (5) By promoting calcium efflux (Frischknecht and Ferrero, 1984).

The first of these actions, inhibition of voltage sensitive calcium channels (VSCC), can be studied in neuronal tissue using isolated synaptic terminals or synaptosomes. As neurotransmitters are released in response to depolarization of the presynaptic terminal, it is reasonable to assume that adenosine could act on VSCCs.

### 3.1.1. Synaptosomes.

To study presynaptic calcium fluxes, a rat brain synaptosomal preparation was used. Synaptosomes, pinched off nerve terminals, are an established preparation that enables the chemical mechanisms of neurotransmission, including membrane ion channels and receptors, to be studied.

Since Gray and Whittaker (1962) first isolated nerve endings from homogenized brain, the viability of the synaptosome as an isolated, working, neuronal compartment has been determined by a vast number of studies. (Reviews: DeBellerocche and Bradford, 1973; Bradford, 1975). During homogenization of neuronal tissue, the nerve terminals are torn from their axons and post synaptic membranes and reseal to form a self contained "package" of viable synaptic terminal components. Electron microscopy studies show the presence of vesicles and mitochondria (Jones and Brearly, 1972). Metabolic respiration at a high and linear rate, and the production of neurotransmitters and amino acids, can be measured (Bradford, 1968; Bradford and Thomas, 1969) which indicates that the complex enzymatic processes of the synaptic terminal are still intact and remain so for a number of hours after preparation.

As synaptic transmission is a calcium dependent process (Augustine et al.,1987), calcium channels and calcium influx in synaptosomes have been studied in detail (Blaustein, 1975; Naschen and Blaustein, 1980; Gripenberg et al.,1980; Scott et al.,1980). The standard method is to use radioactive calcium ( $^{45}\text{Ca}$ ) isotope and to measure its uptake across voltage sensitive calcium channels (VSCC). The channels can be activated by depolarizing the surface membrane. This can be achieved by electrical stimulation via electrodes, by using the alkaloid veratridine to open sodium channels or by increasing the extracellular potassium concentration to alter the potassium diffusion potential. Potassium depolarization provides a simple and rapid method of activating calcium channels. Addition of TTX has no effect on potassium stimulated calcium influx (Blaustein, 1975), indicating there is no influx via opened sodium channels during potassium mediated depolarization.

### 3.1.2. Adenosine and calcium.

The relationship between adenosine and calcium has been shown by a number of studies. Kuroda et al.(1976) demonstrated that adenosine inhibition of field excitatory post synaptic potentials (EPSP) in olfactory cortex could be antagonized by increasing the extracellular calcium concentration in a dose dependent manner. However, similar studies by Dunwiddie (1984) in rat hippocampal slices indicated that, although increases in extracellular calcium did reduce the inhibitory effect of adenosine on the EPSP, this was dependent on the stimulus amplitude and, when the

non-linear summation of EPSPs was taken into account, there was no correlation between inhibition by adenosine and the calcium concentration. A counter argument was put forward by Ribeiro and Sebastiao (1986), who pointed out that postsynaptic effects of adenosine, i.e. hyperpolarization of the postsynaptic membrane, may be partly responsible for the adenosine effects that were independent of the calcium concentration.

Hollins and Stone (1980) postulated that adenosine may act to reduce calcium availability, after observing that adenosine decreased calcium dependent GABA release from potassium stimulated rat cortical slices.

Studies of long term potentiation (LTP) in the hippocampus have shown it to be highly dependent on external calcium; a reduction from 2.4 to 1mM totally abolishing LTP (Dunwiddie and Lynch, 1979). The addition of 2-chloroadenosine also inhibits LTP if given during the high frequency stimulation of the perforant pathway required to induce LTP, but not if given afterwards (Dolphin, 1983). This indicates that the 2-chloroadenosine is inhibiting calcium influx during the stimulus, having the same effect as reducing extracellular calcium.

Schubert et al.(1986) used ion-sensitive microelectrodes to measure changes in extracellular calcium during evoked field potentials in rat hippocampal slices. Synaptic transmission was blocked by lowering calcium to 0.2mM and increasing the magnesium concentration. Pre- and

postsynaptic calcium fluxes could then be differentiated, changes in  $[Ca^{2+}]_e$  being still detectable under these conditions. Repetitive orthodromic stimulation resulted in a decrease in  $[Ca^{2+}]_e$  that was thought to be representative of influx into presynaptic terminals. Addition of 40 $\mu$ M adenosine reduced the size of the calcium signal, i.e. the decrease in  $[Ca^{2+}]_e$  was reduced. When adenosine deaminase was added the calcium signal increased, demonstrating that endogenous adenosine imposed a tonic inhibition on calcium influx.

Studies of calcium channels in single and cultured cells, using voltage and patch clamping techniques, have demonstrated the ability of adenosine to reduce or block calcium channels in a number of tissues including; hippocampal neurones (Proctor and Dunwiddie, 1983; Madison et al., 1987), superior cervical ganglion (Henon and McAfee, 1983) and dorsal root ganglion (Dolphin et al., 1986; Macdonald et al., 1986).

All the aforementioned studies indicate that there is good evidence to show a link between the action of adenosine and calcium availability. However, the most direct approach is to study the calcium channels on nerve terminals in isolation.

A number of reports have demonstrated the inhibitory action of adenosine and related analogues on voltage dependent calcium influx into synaptosomes.



Ribeiro et al.(1979) were the first to show a significant effect of adenosine on calcium influx. They used a crude synaptosomal preparation and were able to show that potassium stimulated (71mM)  $^{45}\text{Ca}$  influx was blocked by adenosine (0.1 - 2mM) over 2 minutes. However, as noted by Fredholm and Hedqvist (1980), the initial 30 seconds showed an increase in influx over control levels at the lower (0.1mM) concentrations. The concentration of adenosine used can also be criticised as being far in excess of that required for maximal inhibition of synaptic transmission as seen in electrophysiological studies.

Wu et al.(1982) confirmed the findings of Ribeiro et al.(1979) and took the study further by demonstrating antagonism of the effect by theophylline, as well as using a range of analogues at pharmacologically acceptable concentrations. They also used a crude synaptosomal preparation, but were careful to show that potassium stimulated (60mM)  $^{45}\text{Ca}$  uptake was confined to synaptosomes only.  $\text{IC}_{50}$  values for adenosine and 2-chloroadenosine were 200nM and 25nM respectively, these ranges being used in the initial experiments reported in this chapter.

Kuroda et al.(1983) presented a brief report of the action of 2-chloroadenosine (50 $\mu\text{M}$ ) on potassium depolarized (30mM)  $^{45}\text{Ca}$  influx into guinea pig cerebral cortex synaptosomes. He demonstrated a small (37%) decrease in calcium uptake measured over 15 seconds that could be antagonized by theophylline.

Arvin et al.(1988) used quin 2 to show that intracellular calcium levels increased in response to a brief pulse of 30mM potassium and this increase could be reduced with 2-chloroadenosine in a dose dependent manner. This effect could be antagonized by theophylline (100 $\mu$ M). However, the use of quin 2 does not allow the origin of the calcium to be determined, so that the increase could have been due to release from intracellular stores, as well as from outside the synaptosome.

A number of reports have also been published that fail to show an effect of adenosine. Barr et al.(1985) used adenosine (10 $\mu$ M) and 2-chloroadenosine (10 - 100 $\mu$ M) at 1 to 15 second uptake times. A significant decrease was seen at 5 seconds uptake, but this was negated by opposing results at 1 and 15 seconds that showed an increase in uptake with both drugs. Most recently, Garritsen et al.(1989) used a range of experimental parameters, including varying preincubation and incubation times, crude and purified synaptosomes, potassium and veratridine depolarization, and addition of adenosine deaminase to remove endogenous adenosine. All failed to show an effect of R-PIA on calcium influx. It would have been interesting to see the effect of other adenosine analogues besides R-PIA.

Michaelis et al.(1988) used a crude P<sub>2</sub> synaptosomal fraction to study <sup>45</sup>Ca influx. They pretreated the synaptosomes with adenosine deaminase and preincubated them

at 37°C for 10 minutes with 2-chloroadenosine (0.01-10µM). Uptake was measured over 20 seconds with 60mM potassium stimulation or 50µM veratridine. They were unable to show any effect on calcium influx. In this case it is possible that the use of adenosine deaminase to remove endogenous adenosine may have had some detrimental effect on the system. In the present study, the use of adenosine deaminase decreased the overall increase in calcium influx, possibly by a non-specific block of channels. A similar observation was reported by Garritsen et al.(1989) who traced the problem to the  $(\text{NH}_4)_2\text{SO}_4$  that the deaminase was suspended in.

### 3.1.3. Sodium / calcium exchange.

As calcium influx via a secondary, and initially unforeseen, route may influence the results obtained in this study, it is appropriate to mention the background to the action of  $\text{Na}^+/\text{Ca}^{2+}$  exchange.

Using a high potassium concentration to depolarize the synaptosomes requires a corresponding decrease in the sodium concentration to maintain the ionic balance. This has led to criticism that  $\text{Na}^+/\text{Ca}^{2+}$  exchange mechanisms across the membrane may contribute to the measured influx of calcium (Coutinho et al, 1984).  $\text{Na}^+/\text{Ca}^{2+}$  exchange is thought to control intrasynaptic calcium levels by removing calcium ions in exchange for sodium (Blaustein and Oborn, 1975). This is dependent on the sodium gradient across the membrane and may also be reversed upon depolarization of the membrane (Coutinho et al, 1984). Synaptosomes maintained in a sodium

rich ACSF tend to have increased intrasynaptic sodium levels that reduce the sodium gradient (Blaustein and Oborn, 1975). When potassium rich ACSF is used to depolarize the synaptosomes, the reduced extracellular sodium further reduces the sodium gradient and may lead to a reversal of  $\text{Na}^+/\text{Ca}^{2+}$  exchange resulting in calcium influx via a route other than VSCC.

Studies carried out have attributed varying importance to this phenomenon. Naschen and Blaustein (1980) attributed less than 15% of the measured calcium influx to being dependent on  $[\text{Na}^+]_e$ . This was found to be constant regardless of the depolarizing potassium concentration, uptake time or calcium concentration. Åkerman and Nicholls (1981) found that verapamil could completely inhibit potassium stimulated  $^{45}\text{Ca}$  influx, indicating that no influx due to  $\text{Na}^+/\text{Ca}^{2+}$  exchange was taking place. If verapamil was also inhibiting  $\text{Na}^+/\text{Ca}^{2+}$  exchange, then an increase in basal  $^{45}\text{Ca}$  levels would be expected due to reduced efflux via the exchanger; this was not observed.

On the other hand, Coutinho et al. (1984) demonstrated that altering  $[\text{Na}^+]_i$  or  $[\text{K}^+]_i$  affected  $^{45}\text{Ca}$  influx. Synaptosomes allowed to attain a high  $[\text{Na}^+]_i$  showed a high  $^{45}\text{Ca}$  influx when depolarized with 150mM potassium, while synaptosomes equilibrated in high potassium ACSF showed a much lower stimulated  $^{45}\text{Ca}$  influx. This is not altogether surprising as 150mM potassium (<5mM sodium) ACSF would result in a very steep sodium gradient out of the

synaptosome, while the synaptosomes preincubated in high potassium would have no membrane potential to induce voltage sensitive calcium influx. They also used the ionophore monensin to destroy the sodium gradient, resulting in a loss of calcium uptake by the synaptosomes.

However, studies by Suszkiew et al.(1986) give more convincing evidence as to the contribution of  $\text{Na}^+/\text{Ca}^{2+}$  exchange to calcium influx. Comparison of synaptosomes resuspended in normal (140mM) sodium or in ACSF with choline replacing the sodium, showed a large increase in  $[\text{Na}^+]_i$  of 77mM immediately upon resuspension of the synaptosomes in the ACSF. This increased to 104mM after 30 minutes, as compared to a maximal increase of 5mM sodium into synaptosomes resuspended in the choline ACSF. Also, the membrane potential between the two preparations varied by 26mv (-78mv to -52mv). However, there was little difference in  $[\text{Ca}^{2+}]_i$  between them, as determined by quin 2, indicating the calcium gradient was not upset to any great extent in basal (resting) synaptosomes. Upon depolarization with 52mM potassium a greater increase in  $^{45}\text{Ca}$  influx was observed in the sodium rich synaptosomes.

These experiments demonstrate that the sodium gradient across the synaptosomal membrane must be taken into account when calcium influx is quantified. However, the use of potassium rich ACSF, with potassium totally replacing sodium, as used in some experiments, greatly exaggerates the

problem. Although there is a component of  $\text{Ca}^{2+}$  influx attributable to  $\text{Na}^+/\text{Ca}^{2+}$  exchange, if a shallow sodium gradient (70mM) is used, and preincubation of the synaptosomes in sodium rich (basal) ACSF is reduced to a minimum (20 minutes), then this factor can be reduced.

As the existing studies, on the effect of adenosine on synaptosomal calcium flux, fail to show a clear cut or consistent action of adenosine, it was decided to carry out a detailed study. The following experiments attempt to determine the optimum conditions for measuring calcium influx and look at the effect of a range of adenosine analogues on synaptosomal  $^{45}\text{Ca}$  influx. In addition the effect of adenosine on calcium influx into hippocampal slices is studied to determine if the action of adenosine is apparent in a more intact preparation and to tie in with the electrophysiological studies reported in the preceding chapters.

### 3.2. MATERIALS AND METHODS

The experiments reported in this chapter were carried out over two limited time periods of three and four months spent in the labs of Dr. M.G.Wyllie at Pfizer Central Research, Sandwich, Kent.

#### 3.2.1. Compounds.

Analar grade compounds were obtained from BDH Chemicals Ltd. U.K.. Lanthanum chloride, adenosine hemisulphate and adenosine deaminase (Type II, powder) were obtained from Sigma Chemical Co., U.K.. Adenosine agonists and antagonists were from RBI (Semat Technical Ltd, U.K.).  $^{45}\text{CaCl}_2$  was from Amersham International PLC, U.K.. Scintillation fluids were from Packard or National Diagnostics.

CHA and CPA were dissolved in ethanol and R and S-PIA were dissolved in DMSO to give a stock solution. The final concentration of vehicle was never more than 0.5%. All other compounds were dissolved in basal ACSF to give the desired concentration when added to the incubation volume.

### 3.2.2. Experimental solutions.

The synaptosomal basal ACSF was composed of:

NaCl, 135mM; KCl, 5mM; Tris HCl, 20mM (pH 7.4 at 37°C);  
MgCl<sub>2</sub>.6H<sub>2</sub>O, 1.2mM; CaCl<sub>2</sub>.6H<sub>2</sub>O, 1.25mM; D-glucose, 10mM;  
saturated with O<sub>2</sub>.

This solution was used in all basal test experiments and to resuspend the synaptosomes. The depolarizing ACSF was adjusted to give 70 mM KCl, 70mM NaCl.

The lanthanum stop solution contained:

NaCl, 135mM; KCl, 5mM; Tris HCl, 20mM (pH 7.4 at 5°C);  
LaCl<sub>3</sub>, 1mM.

The hippocampal slice basal ACSF contained:

KH<sub>2</sub>PO<sub>4</sub>, 2.2mM; MgCl<sub>2</sub>.6H<sub>2</sub>O, 1.2mM; D-glucose, 10mM;  
NaHCO<sub>3</sub>, 25mM; NaCl, 115mM, gassed with 95%O<sub>2</sub>/5%CO<sub>2</sub>, pH 7.4.

The hippocampal slice lanthanum stop solution contained:

NaCl, 135mM; KCl, 5mM; HEPES, 20mM; MgCl<sub>2</sub>.6H<sub>2</sub>O, 1.2mM;  
LaCl<sub>3</sub>, 10mM.

The dissolving fluid for the hippocampal slices consisted of 36g NaOH (3M), 150mls methanol and 45mls Triton X-100 in 300mls water.



### 3.2.3. Preparation of synaptosomes.

All procedures were carried out at 5°C. Two male Sprague-Dawley rats (250g) were used per experiment.

The animals were killed by stunning and cervical dislocation and the brains dissected out into ice cold 0.32M sucrose plus 20mM tris, pH 7.4 at 5°C.

The brains were chopped manually using scissors and then homogenized. A Potter S homogenizer (B.Braun) with a loose fitting pestle and mortar was used to homogenize the tissue with five steady up and down strokes at 850 rpm.

The homogenate was then centrifuged and passed through a discontinuous density sucrose gradient to produce a purified synaptosomal fraction. Details are shown in fig. 3.0.

The synaptosomes were used immediately after preparation, or held on ice in pellet form if necessary, to reduce the influx of sodium that may upset the  $\text{Na}^+/\text{Ca}^{2+}$  exchange (see section 3.3.5).

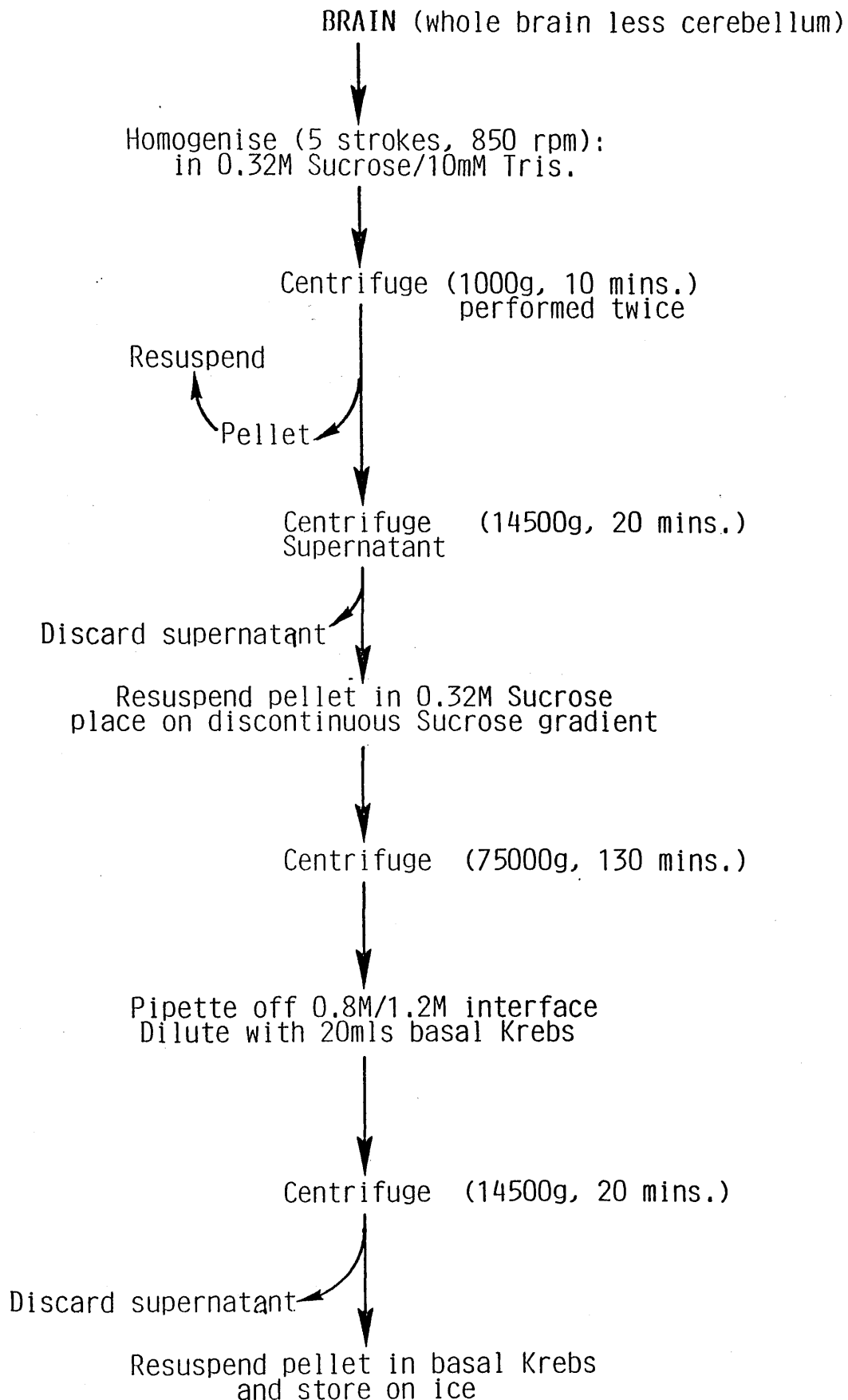


Fig. 3.0. Flow diagram of the synaptosome preparation.

#### 3.2.4. Synaptosome viability.

The viability of the synaptosomes was confirmed by the ability of potassium to depolarize the synaptosomes, as was apparent by the increased calcium influx.

When the preparation was originally set up in the Pfizer laboratories, a series of experiments was carried out to ensure the preparation technique could produce viable synaptosomes. Measurement of oxygen levels using  $O_2$  electrodes showed an increase in the rate of oxygen consumption upon addition of veratridine or potassium when glucose was present. This was taken to show that the components of the purified synaptosomal fraction were metabolically active and the activity increased upon depolarization of the synaptosomes.

### 3.2.5. Measurement of calcium uptake into synaptosomes.

Test solutions were of 1ml volume consisting of 50µl  $^{45}\text{CaCl}_2$  (1µCi), 50µl drug or vehicle and 850µl basal or depolarizing ACSF. All drug concentrations were performed in triplicate.

The tubes were placed in racks in a water bath at 37°C and allowed to equilibrate. Uptake was started by the addition of 50µl synaptosomes using an Eppendorf repeat dispenser pipette. The synaptosomes were preincubated with the compounds and prewarmed to 37°C for 10 minutes unless stated otherwise. After the required incubation time, uptake was stopped by using a lanthanum stop buffer (3mls).

Lanthanum is a potent calcium channel blocking metal ion which rapidly blocks calcium influx and efflux. It also displaces externally bound calcium over 15-30 minutes (Jansson et al., 1977).

The tubes were placed on ice for 20 minutes to allow the lanthanum to displace any externally bound calcium. The solutions were then filtered through presoaked GFB filters and washed with approximately 3x3mls of lanthanum stop buffer using an Ilicon cell harvester. The filters were placed in scintillation vials (skatron) with 3mls of Ecoscint scintillation fluid and counted for 1 minute in an LKB β counter.

To account for any passive uptake or remaining externally bound calcium, blank values were determined by adding synaptosomes to tubes already containing 3mls of lanthanum stop buffer.

### 3.2.6. Measurement of 1 second calcium uptake.

A modified procedure was used to measure calcium influx over 1 second to ensure maximum possible mixing of the synaptosomes with  $^{45}\text{Ca}$  and drug.

ACSF - Tris buffers were the same as for longer uptake experiments except for the depolarizing ACSF where the NaCl was totally replaced with KCl (140mM).

The incubation volume was 0.5mls. 50 $\mu$ l of drug or vehicle in basal ACSF was added to 200 $\mu$ l of synaptosomes in basal ACSF. The tubes were then preincubated for 10 mins at 37°C.

$^{45}\text{CaCl}_2$  stock was added to aliquots of basal (5mM  $\text{K}^+$ ) or depolarizing (140mM  $\text{K}^+$ ) ACSF to give a final activity of 2 $\mu$ Ci/ml, and prewarmed to 37°C.

The 1 second preincubation period was started by the addition of 250 $\mu$ l of basal or depolarizing ACSF using Eppendorf repeat dispenser pipettes. The 140mM  $\text{K}^+$  mixed to give a final concentration of 72.5mM  $\text{K}^+$ . Uptake was terminated by the addition of 2mls iced lanthanum stop buffer from a 50ml Eppendorf. A metronome was used to maintain a rhythm of addition as close to 1 second as possible.

The action of pipetting the  $^{45}\text{Ca}$  containing ACSF directly onto the synaptosomes ensures thorough mixing of the solution and this, together with the larger volume of synaptosomes and increased activity, ensures good counts can be obtained.

### 3.2.7. Measurement of calcium uptake into hippocampal slices.

Hippocampal slices were prepared from Sprague-Dawley rats of either sex weighing approximately 250g as described in section 1.2.5. The slices were incubated at room temperature for 1 hour, then cut to a standard size using a cut pipette tip (internal diameter 4.5mm) and left for a further 30 mins. A calcium free ACSF was used during the preparation to deplete the intracellular calcium and give a greater stimulated uptake (Retz and Coyle, 1984), as determined by preliminary studies.

The experiments were carried out in 24 well culture plates (Falcon). Basal and test incubations were carried out in triplicate. Slices were preincubated in 1ml of basal ACSF plus drug for 10 mins at 37°C. 1 $\mu$ Ci/ml  $^{45}$ CaCl<sub>2</sub> and 0.625mM CaCl<sub>2</sub> was added to the ACSF.

1ml of basal (2.2mM K<sup>+</sup>) or depolarizing (70mM K<sup>+</sup>) ACSF was added to the wells together with the appropriate drug or drug vehicle. After preincubation, slices were tipped into a plastic petri dish and a single slice was added to each well using a sieve spatula (Pfizer workshops). The slices were incubated for 10 minutes at 37°C.

Uptake was terminated by transferring the slice by pasteur pipette through two 100ml beakers of ice cold 10mM lanthanum HEPES buffered ACSF and then to 5mls of cold lanthanum ACSF for 1hr. HEPES was used, as lanthanum cannot be added to bicarbonate buffered ACSF due to precipitation problems.

After 1hr, the slices were removed, drained on filter paper, weighed and transferred to scintillation vials (Beckman) containing 0.25mls of dissolving fluid. The tubes were incubated at 50°C for 10 minutes after which the vials were vortexed, 2mls of Hionic Fluor scintillation fluid (Packard) added, and the vials capped and vortexed before being counted on a Beckman scintillation counter.

### 3.2.8. Determination of calcium uptake in nmol<sup>45</sup>Ca/mg protein and DPM/wet wt. tissue.

Values of calcium uptake into the synaptosomes were determined by the following calculation:

$$\frac{\left( \frac{\text{Depolarized} - \text{Basal}}{\text{total DPM} / 1000} \right)}{\text{mg protein}}$$

Total DPM was determined by counting 50µl of <sup>45</sup>Ca. The protein concentration of a sample of the synaptosomal stock was determined by Bio-rad protein assay.

Calcium uptake for the slices was measured in DPM/mg wet weight tissue.

After being touched with a piece of filter paper to draw off excess fluid, the individual slices were placed on a piece of preweighed foil and weighed on a Sartorius 7 figure balance before being transferred to the scintillation vial. The DPM values from each tube were divided by the weight to give the DPM in mg wet weight of tissue

### 3.2.9. Protein determination.

100µl samples of the synaptosome stock were diluted 1 in 10, 100µl of 0.1M NaOH added and incubated at 50°C for 10 mins to break down the tissue. 100µl was then mixed with 5mls of Bio-Rad (Bradford) reagent, left for 10 minutes and the absorbance measured at 595nm on an LKB spectrometer.



### 3.3. RESULTS

#### 3.3.1. Determination of the optimum parameters for studying calcium uptake.

A series of experiments were carried out to determine the optimum parameters for studying calcium uptake into synaptosomes.

##### 3.3.1 (i). Potassium concentration.

To determine a suitable potassium concentration for depolarization of the synaptosomes, the uptake of calcium was measured at increasing extracellular potassium concentrations over the three time periods used in these studies (Fig.3.1.).

The  $EC_{50}$  values determined for each uptake time were 50mM for 120 secs, 55mM for 20 secs and 50mM for 1 sec.

A concentration of 50mM potassium was used in some initial experiments, but a more consistent increase in uptake was found to be obtained when a slightly higher potassium concentration was used, particularly at 20 second uptake periods.

It was therefore decided to use a potassium concentration of 70mM to ensure rapid depolarization.

Fig. 3.1. Potassium concentration effect curves for stimulation of calcium uptake into synaptosomes.

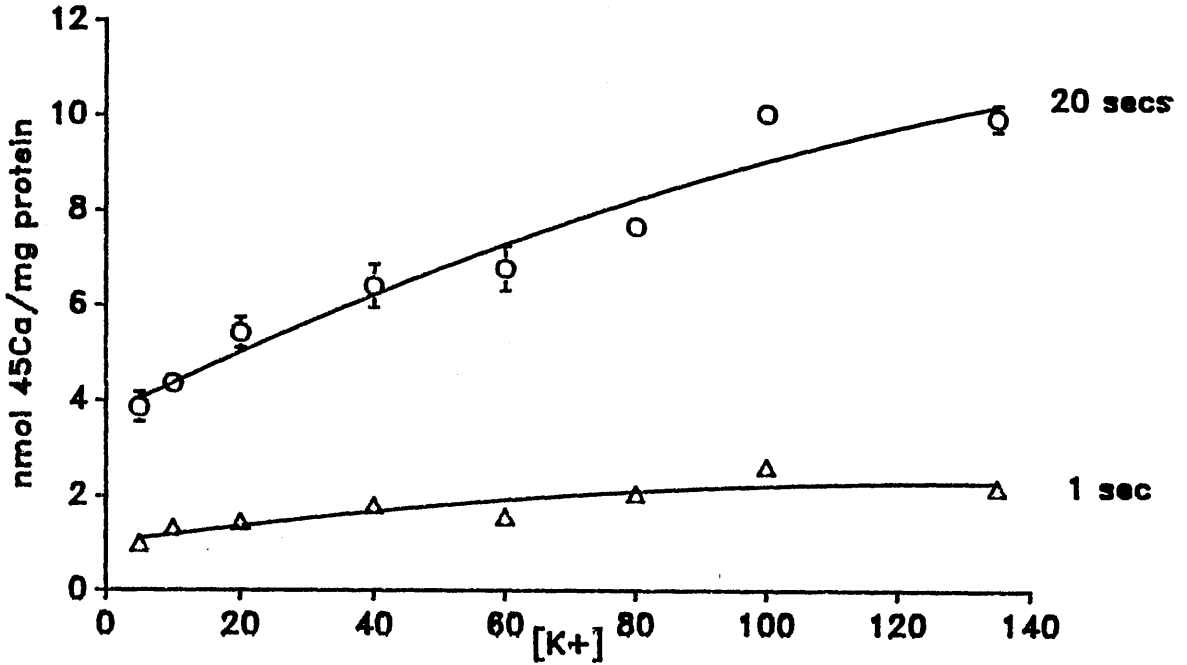


Fig. 3.1a. Determination of synaptosomal  $^{45}\text{Ca}$  uptake measured in nmol/mg protein at increasing extracellular potassium concentrations. Uptake was measured over 1 and 20 secs. Points are mean  $\pm$  sem of 6 determinations. Synaptosomes were prewarmed before addition.

The  $\text{EC}_{50}$  values for each curve are 55mM for 20 secs and 50mM for 1 sec.

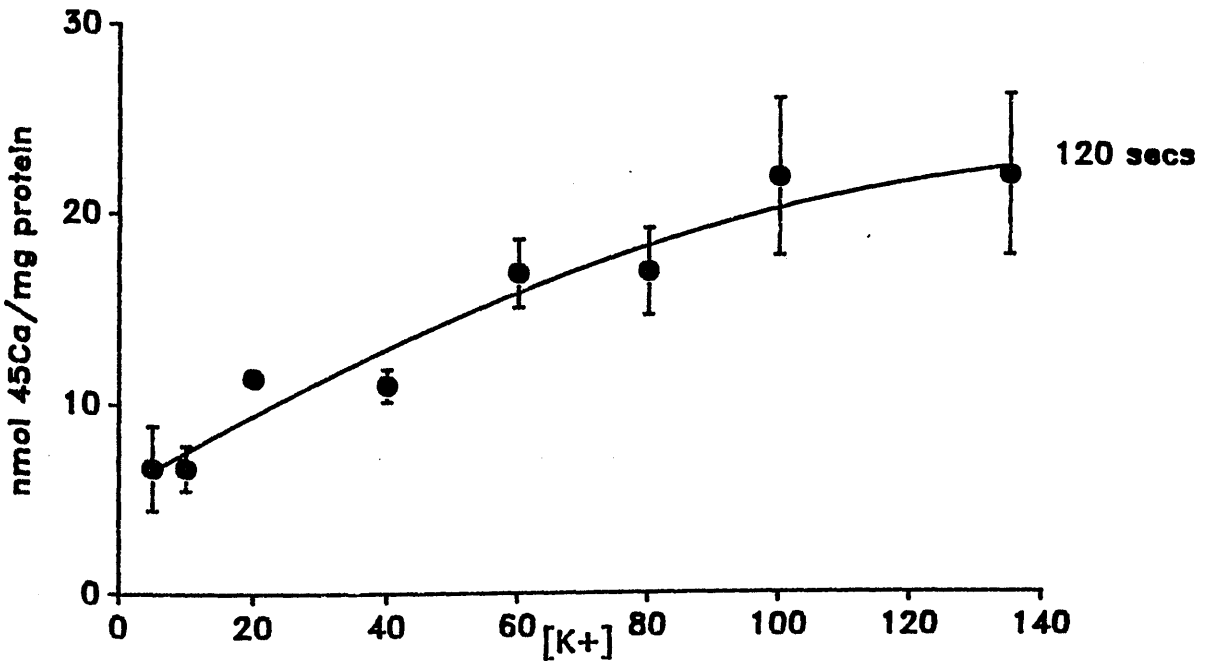


Fig. 3.1b. Determination of synaptosomal  $^{45}\text{Ca}$  uptake at increasing extracellular potassium concentrations. Uptake was measured over 120 secs. Points are mean  $\pm$  sem of 6 determinations. Synaptosomes were prewarmed before addition. The  $\text{EC}_{50}$  value is 50mM.

### 3.3.1 (ii). Uptake of calcium over time.

Calcium uptake over increasing time periods was measured (Fig. 3.2.).

Maximum depolarized uptake was reached after 30 seconds and trailed off over longer periods up to 2 minutes. Basal uptake also increased over 30 seconds and continued to rise over 2 minutes (Fig. 3.2a). An optimal uptake period was taken as 20 seconds as this gave maximal stimulated uptake after basal uptake had been subtracted.

Fig. 3.2. Time response curves for potassium stimulated calcium uptake into synaptosomes.

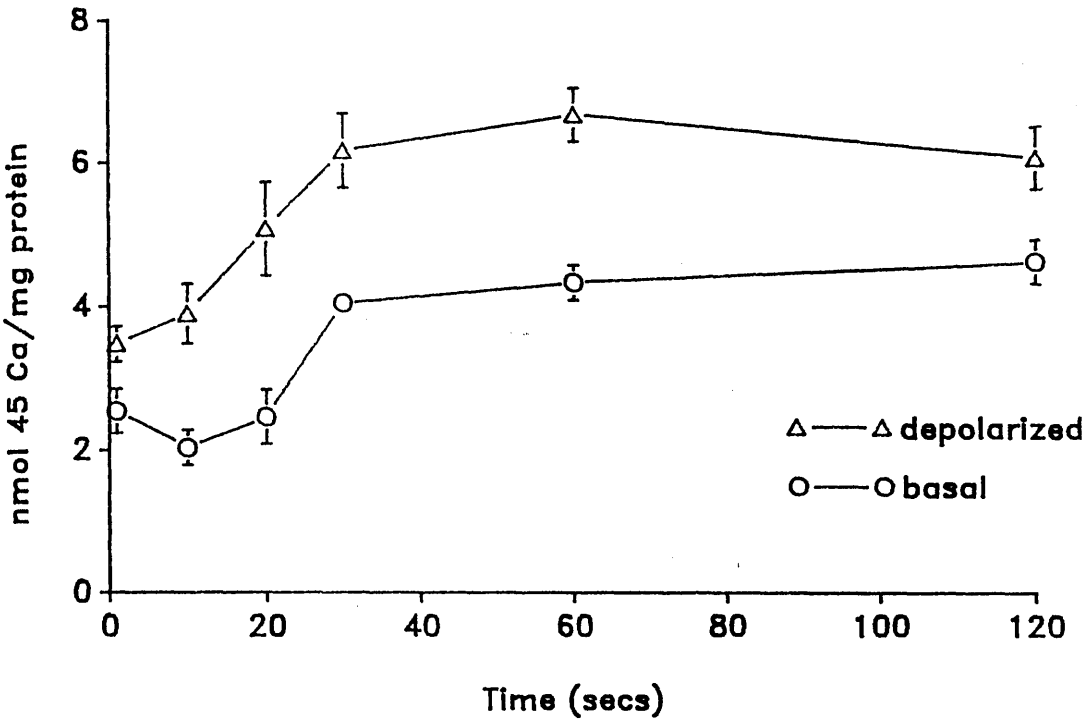


Fig. 3.2a. Basal (5mM  $\text{K}^+$ ) and depolarized (70mM  $\text{K}^+$ ) calcium uptake into synaptosomes measured over increasing time periods.

Points are mean  $\pm$  sem of 6 determinations. Potassium concentration was 70mM, synaptosomes were prewarmed at 37°C.

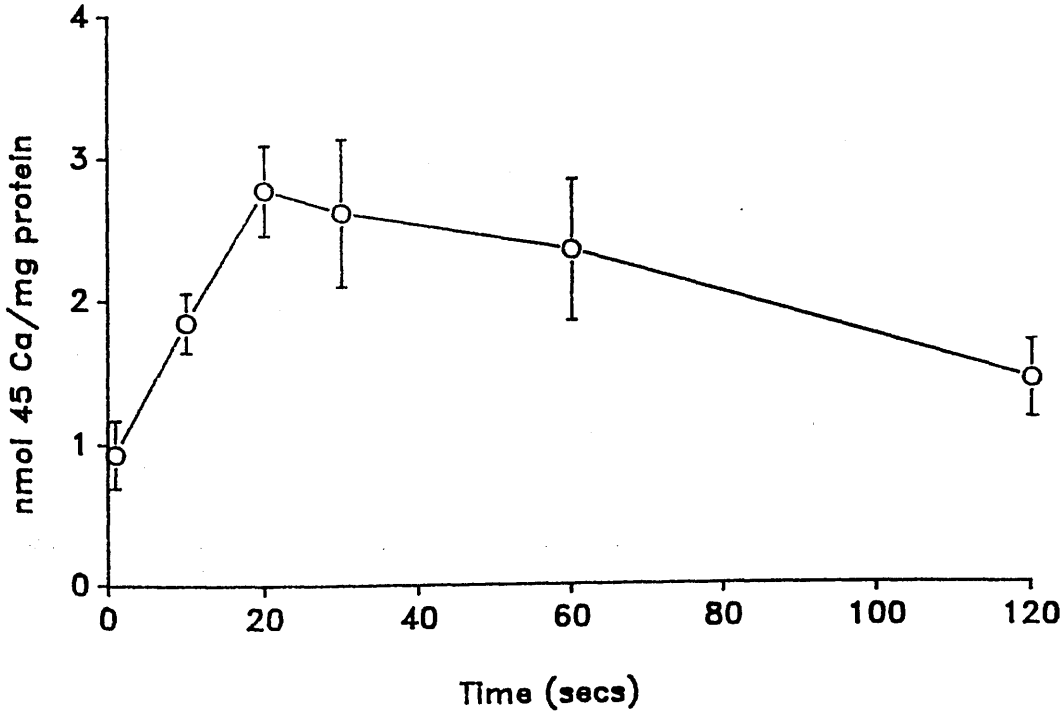


Fig. 3.2b. Stimulated calcium uptake (depolarized minus basal) over time. Data calculated from graph above.

### 3.3.2. Experimental conditions

In all subsequent experiments, unless otherwise stated, the synaptosomes were depolarized with 70mM potassium and the results expressed as stimulated uptake, i.e. the difference between basal and depolarized uptake. Uptake is calculated in nmol  $^{45}\text{Ca}$ /mg protein for the synaptosomes and DPM/mg wet weight tissue for the hippocampal slices. (See methods for calculation of values.)

### 3.3.3. Inhibition of calcium uptake by metal ions

Divalent cations, particularly certain transition metal ions, bind to calcium channels and block the passage of calcium through the channel (Naschen, 1984). To determine the viability of the synaptosomes and to show that uptake across voltage sensitive calcium channels could be blocked, dose response curves to cadmium, cobalt and nickel were determined (Figs. 3.3-3.4).

Cadmium and nickel are thought to differentiate between the different proposed calcium channel subtypes (Tsien et al., 1987). N and L channels, associated with neurones, are blocked by cadmium and resistant to nickel while T channels are blocked by nickel. This is reflected in the relative potency of cadmium and nickel in blocking uptake. The  $\text{IC}_{50}$  values are 15 $\mu\text{M}$  for cadmium and 400 $\mu\text{M}$  for nickel at 2 minutes uptake. Cobalt was used to show the calcium channel blocking properties of this compound to support its use in the magnesium replacement experiments in chapter 1.

Fig. 3.3. The effect of cadmium on synaptosomal calcium uptake.

Dose response curves to cadmium at 1, 20 and 120 secs were determined. For all experiments, metal ions were added directly to the tubes and synaptosomes were prewarmed for 10 mins at 37°C.

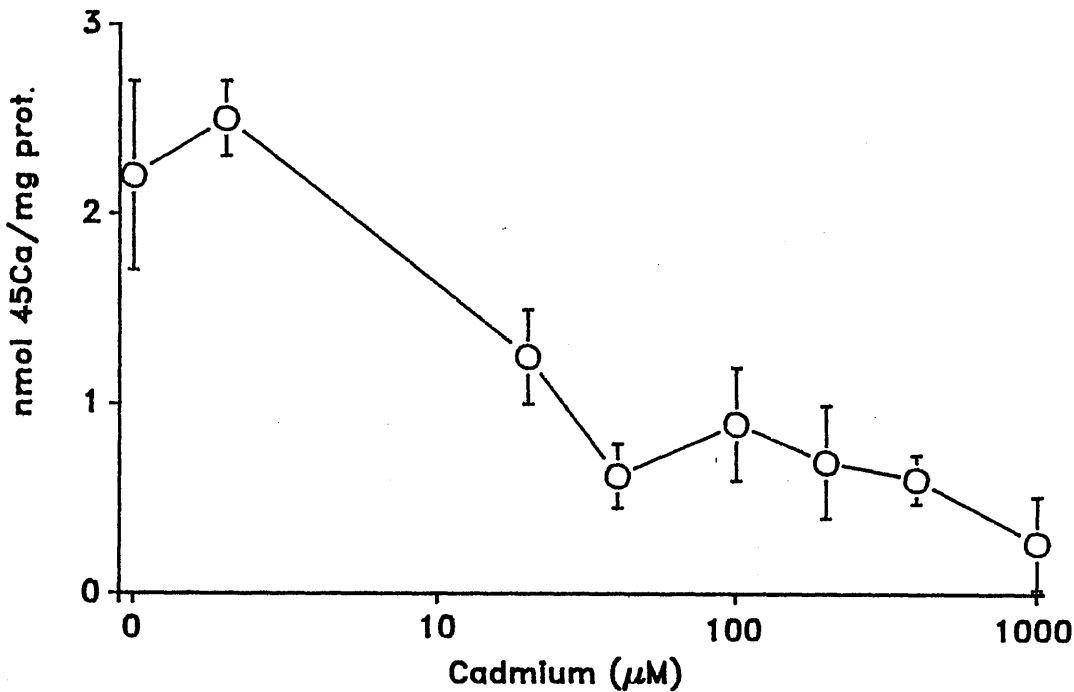


Fig. 3.3a. The effect of increasing concentrations of cadmium on stimulated calcium uptake measured over 120 secs. Points are mean  $\pm$  sem of 6 determinations. The  $IC_{50}$  value is 15  $\mu$ M.

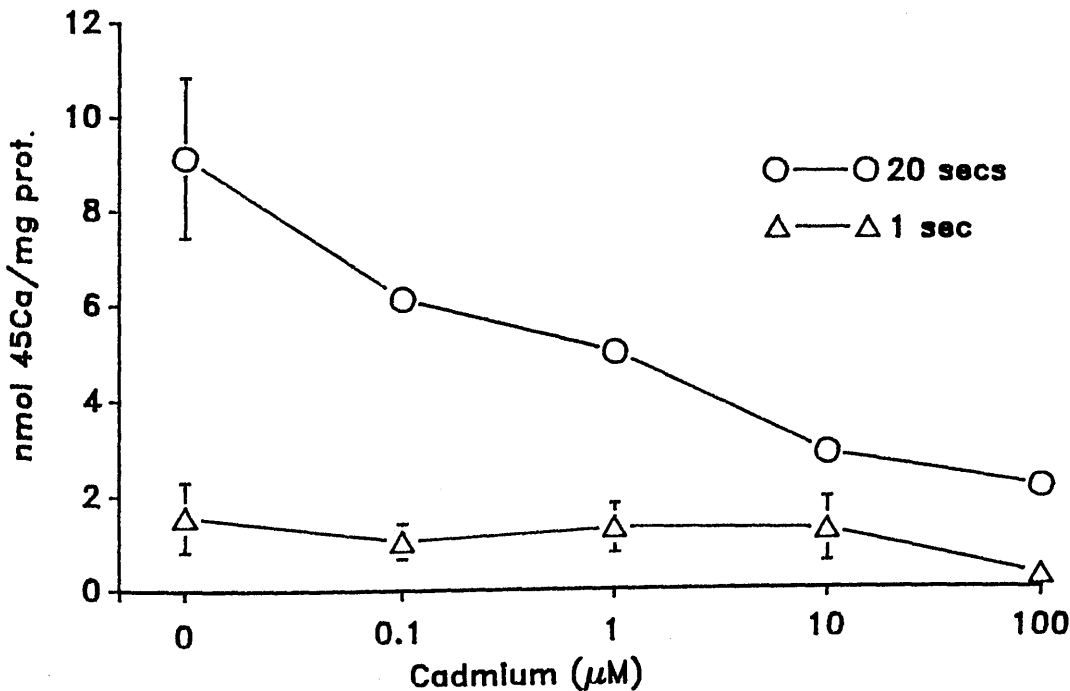


Fig. 3.3b. The effect of increasing concentrations of cadmium on stimulated calcium uptake measured over 1 and 20 secs. Points are mean  $\pm$  sem of 3-6 determinations. The  $IC_{50}$  values are 0.3  $\mu$ M for 20 secs and 35  $\mu$ M for 1 sec.

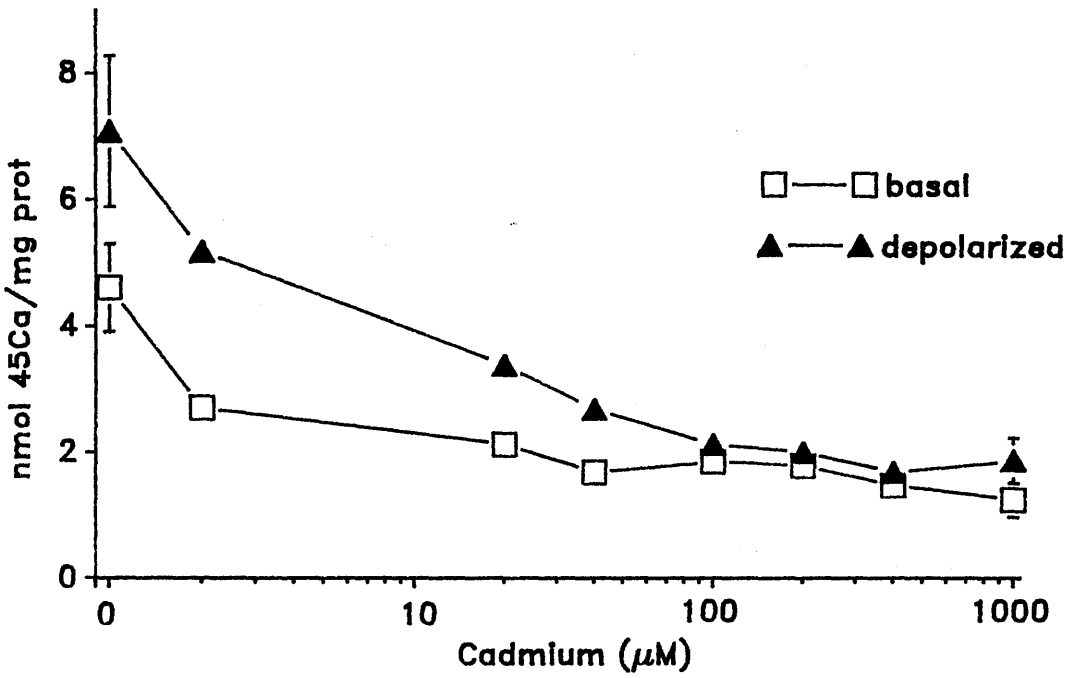


Fig.3.3c shows the data for 3.3a as basal and depolarized calcium uptake. This shows that passive calcium influx is also reduced, cadmium tending to block all sites of calcium influx.

Fig.3.4. The effect of nickel and cobalt on synaptosomal calcium uptake.

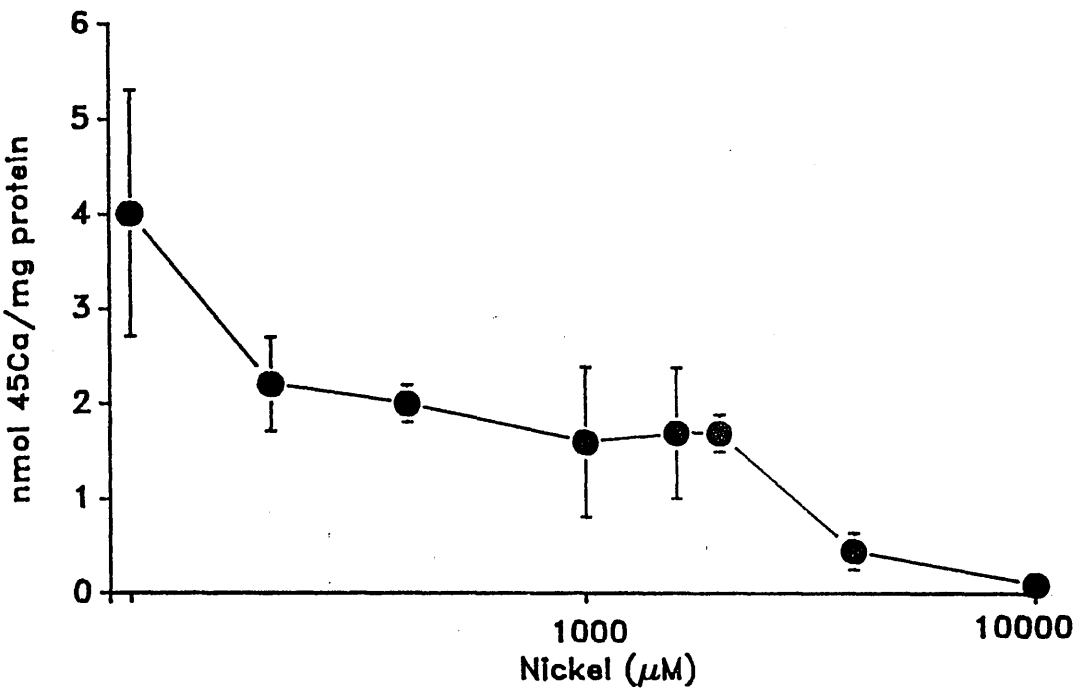
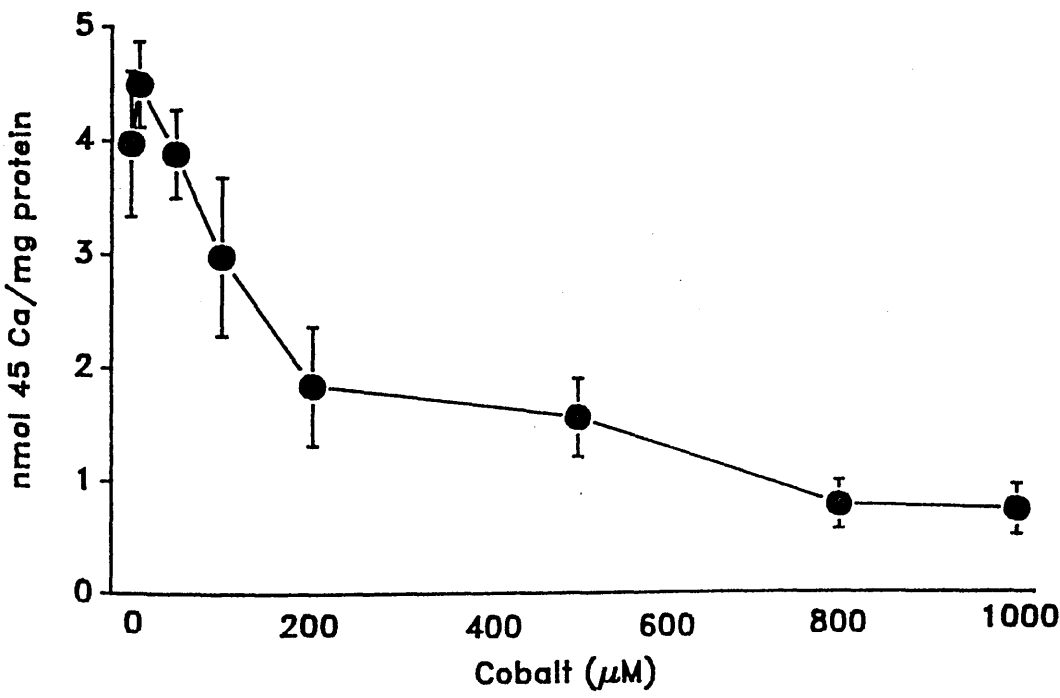


Fig. 3.4a. Inhibition of stimulated calcium uptake by nickel. Uptake was measured over 2 minutes. Points are mean  $\pm$  sem of 6 determinations. Synaptosomes were prewarmed for 10 mins at 37°C.  $IC_{50} = 400\mu M$ ,



Figs. 3.4b. Inhibition of stimulated calcium uptake by cobalt. Uptake was measured over 2 minutes. Points are mean  $\pm$  sem of 6 determinations. Synaptosomes were prewarmed for 10 mins at 37°C.  $IC_{50} = 130\mu M$



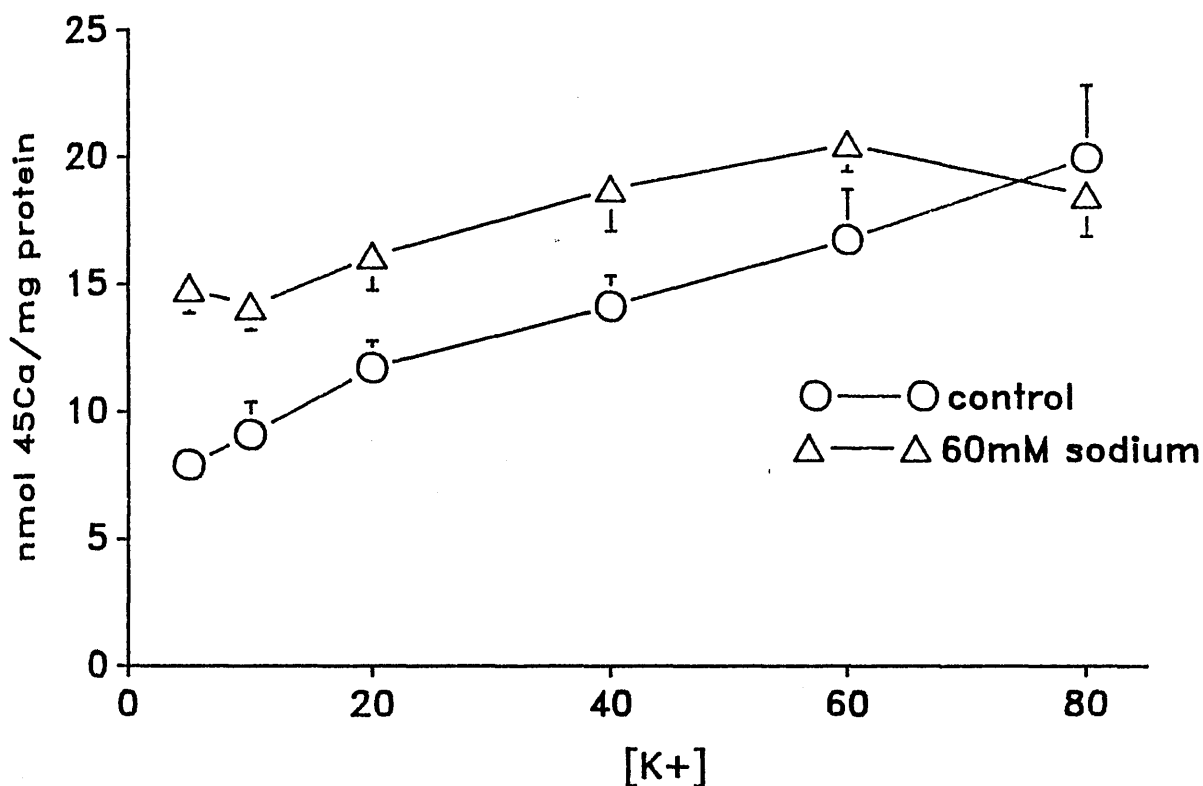
#### 3.3.4. Sodium/calcium exchange

As discussed in the introduction to this chapter, a component of the observed calcium uptake may occur via the  $\text{Na}^+/\text{Ca}^{2+}$  exchange mechanism. An idea of the degree of calcium uptake from  $\text{Na}^+/\text{Ca}^{2+}$  exchange can be determined by the following experiment.

Dose response curves to potassium were determined in the presence of variable or fixed (60mM) sodium. For the control (variable) curve, the sodium concentration was reduced as the potassium was increased to maintain the ionic balance at 140mM  $\text{Na}^+$  plus  $\text{K}^+$ . For the "fixed sodium" curve, sodium was maintained at 60mM and the ionic balance was maintained by adding choline chloride.

It is apparent from the graph (Fig.3.5.) that calcium uptake is increased by low extracellular sodium. At 5mM  $\text{K}^+$  there is an 87% increase in calcium uptake which is presumably due to uptake across the  $\text{Na}^+/\text{Ca}^{2+}$  exchange. If the level of stimulated uptake at 80mM  $\text{K}^+$  is taken as 100%, then 56% of the total uptake can be accounted for by passive  $\text{Na}^+/\text{Ca}^{2+}$  influx. However, as the gradient of the two lines remains roughly constant, it is reasonable to assume that the other 44% is due to voltage sensitive calcium channel activated uptake or at least not via a sodium gradient dependent uptake.

Fig. 3.5. The effect of a reduced concentration of sodium on potassium stimulated calcium uptake into synaptosomes.



The effect of a reduced ACSF sodium concentration on synaptosomal calcium uptake at increasing potassium concentrations. The synaptosomes were preincubated in basal ACSF at  $37^\circ\text{C}$  and uptake was determined over 120 secs. Points are mean  $\pm$  sem of 6 determinations.

### 3.3.5. The action of adenosine and adenosine analogues on synaptosomal calcium uptake.

The effect of adenosine and six adenosine analogues on calcium uptake across voltage sensitive calcium channels was determined.

In preliminary studies a range of experimental conditions was tried to see if uptake could be inhibited in sub-optimal conditions.

#### 3.3.5.(i) Direct addition of synaptosomes to the test solutions.

If adenosine or its analogues can act to block calcium uptake by a non-specific route, such as direct block of channels or chelation of calcium, then direct addition of the synaptosomes to test solutions containing the drugs should be enough to reduce the uptake of calcium.

The data in table 3.1. shows that calcium uptake was unaffected by adenosine or its analogues under these conditions.

Compound	Control	Concentration $\mu\text{M}$							
		0.001	0.01	0.05	0.1	0.2	1	5	
L-PIA	$3.8 \pm 1.22$	-	$3.0 \pm 0.75$	-	$4.3 \pm 0.93$	$3.0 \pm 0.4$	$3.9 \pm 0.4$	-	
D-PIA	$3.7 \pm 0.71$	-	$2.6 \pm 0.44$	$4.0 \pm 1.55$	$3.3 \pm 0.74$	-	$4.2 \pm 0.47$	$3.0 \pm 1.42$	
NECA	$2.7 \pm 0.37$	-	-	$2.4 \pm 0.3$	-	$2.7 \pm 0.65$	$2.7 \pm 0.15$	-	
CHA	$4.0 \pm 0.6$	$3.1 \pm 1.3$	$4.1 \pm 1.25$	-	$3.6 \pm 1.0$	-	$3.9 \pm 0.2$	-	
CPA	$3.2 \pm 0.55$	$2.5 \pm 0.5$	$2.8 \pm 0.46$	-	$2.4 \pm 0.2$	-	-	-	
	Control	10	20	50	100	200	500		
Adenosine	$2.2 \pm 0.71$	$2.2 \pm 0.38$	$2.0 \pm 0.62$	$2.8 \pm 0.56$	$2.1 \pm 0.46$	$2.1 \pm 0.07$	$2.2 \pm 0.45$		

Table 3.1 Calcium uptake in nmol  $^{45}\text{Ca}/\text{mg}$  protein. The synaptosomes were kept on ice prior to experimentation. Drugs were added directly to the tubes. Uptake was over 2 minutes at  $37^\circ\text{C}$ . Values are mean  $\pm$  sem of 3 determinations. There was no significant difference between control and test values ( $P > 0.1$ , paired t-test.).

3.3.5.(ii). Preincubation of synaptosomes with the adenosine compounds.

As adenosine is known to act via a receptor, preincubation of the synaptosomes with the drugs should allow binding to a maximum number of receptor sites. The receptors should then be activated upon depolarization of the membrane.

The data in table 3.2. shows that the adenosine compounds had no significant or consistent effect on calcium uptake. Two values were significantly different from control values (\* =  $P < 0.1$ , paired t-test.).

Compound	Concentration $\mu\text{M}$					
	Control	0.1	1	50	100	
(a) 2 min						
Adenosine	$4.9 \pm 0.3$	-	-	$3.6 \pm 0.3$	$4.3 \pm 0.2$	
NECA	$8.7 \pm 1.83$	$4.0 \pm 0.44$	$7.6 \pm 0.22$	-	-	
D-PIA	$9.5 \pm 0.7$	$8.4 \pm 0.36^*$	$11.0 \pm 0.54$	-	-	
(b) 20 s						
	Control	1	2	5	10	
NECA	$5.1 \pm 0.5$	$3.8 \pm 0.5$	-	$3.7 \pm 0.4$	-	
L-PIA	$10.3 \pm 0.05$	-	-	$13.6 \pm 1.2$	$14.0 \pm 0.85^*$	
CADO	$11.7 \pm 0.08$	-	$9.0 \pm 0.7$	-	$11.6 \pm 0.5$	
CHA	$4.7 \pm 0.2$	$5.1 \pm 0.8$	-	$4.5 \pm 0.09$	-	
(c) 1 s						
	Control	2	5	10	50	100
Adenosine	$2.2 \pm 0.5$	-	-	-	$1.26 \pm 0.92$	$3.0 \pm 1.05$
L-PIA	$3.6 \pm 0.9$	-	$3.2 \pm 0.5$	$3.15 \pm 0.45$	-	-
CADO	$1.75 \pm 0.05$	$2.1 \pm 0.4$	-	$1.7 \pm 0.7$	-	-

Table 3.2 Calcium uptake in nmol  $^{45}\text{Ca}$ /mg protein. The synaptosomes were preincubated with the drugs for 30 minutes on ice. Incubation times were for 1, 20 and 120 seconds. Values are mean  $\pm$  sem of 3 determinations.

In most cases, there was no significant difference between control and test values (\* =  $P < 0.1$ , paired t-test).

### 3.3.6. Preincubation and prewarming of synaptosomes with the adenosine compounds.

The receptor to which adenosine binds is thought to act via G-proteins to exert an effect on ion channels (Fredholm and Dunwiddie, 1988). If this is the case then prewarming of the synaptosomes will allow biochemical pathways to be reactivated after the preparation process and allow adenosine to exert an effect.

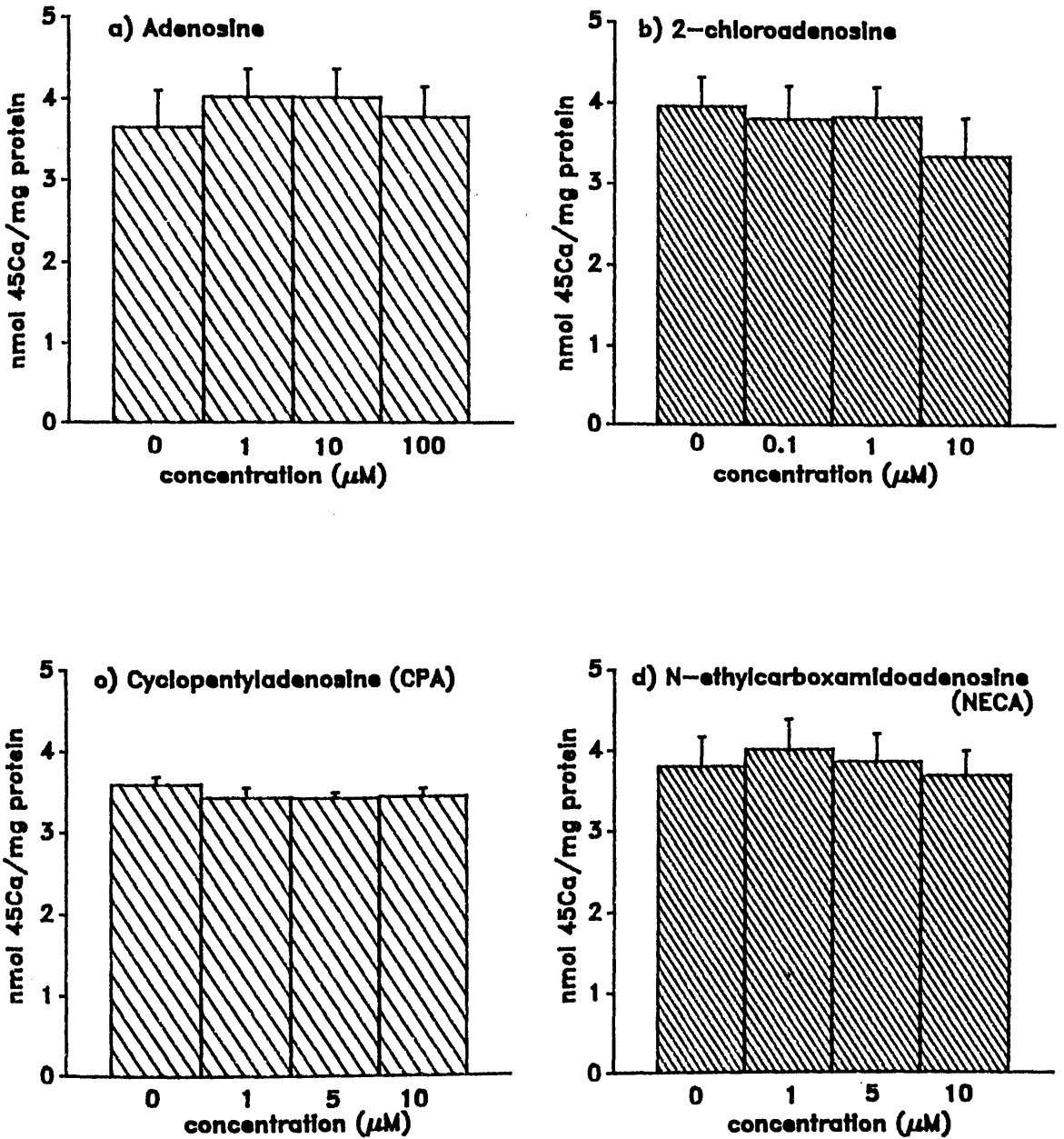
The synaptosomes were preincubated with the adenosine compounds for 10 minutes at 37°C prior to experimentation and calcium uptake measured over 1 and 20 seconds (Figs. 3.6 and 3.7).

Optimal parameters were determined as 20 seconds uptake with preincubation and prewarming of the synaptosomes with the adenosine analogues.

The adenosine analogues 2-chloroadenosine and NECA showed a small but significant inhibition of the stimulated calcium uptake measured over 20 second uptake periods. The maximum inhibition was a 32% reduction in calcium uptake by 2-chloroadenosine ( $P < 0.01$ , paired t-test.). CHA and CPA also showed significant decreases in calcium uptake at low concentrations but not at higher concentrations.

The small but significant decrease in calcium uptake by some of the adenosine analogues was apparent in the depolarized synaptosomes only (Fig 3.8.) indicating that inhibition of the voltage activated calcium channels was responsible for the decreased calcium uptake.

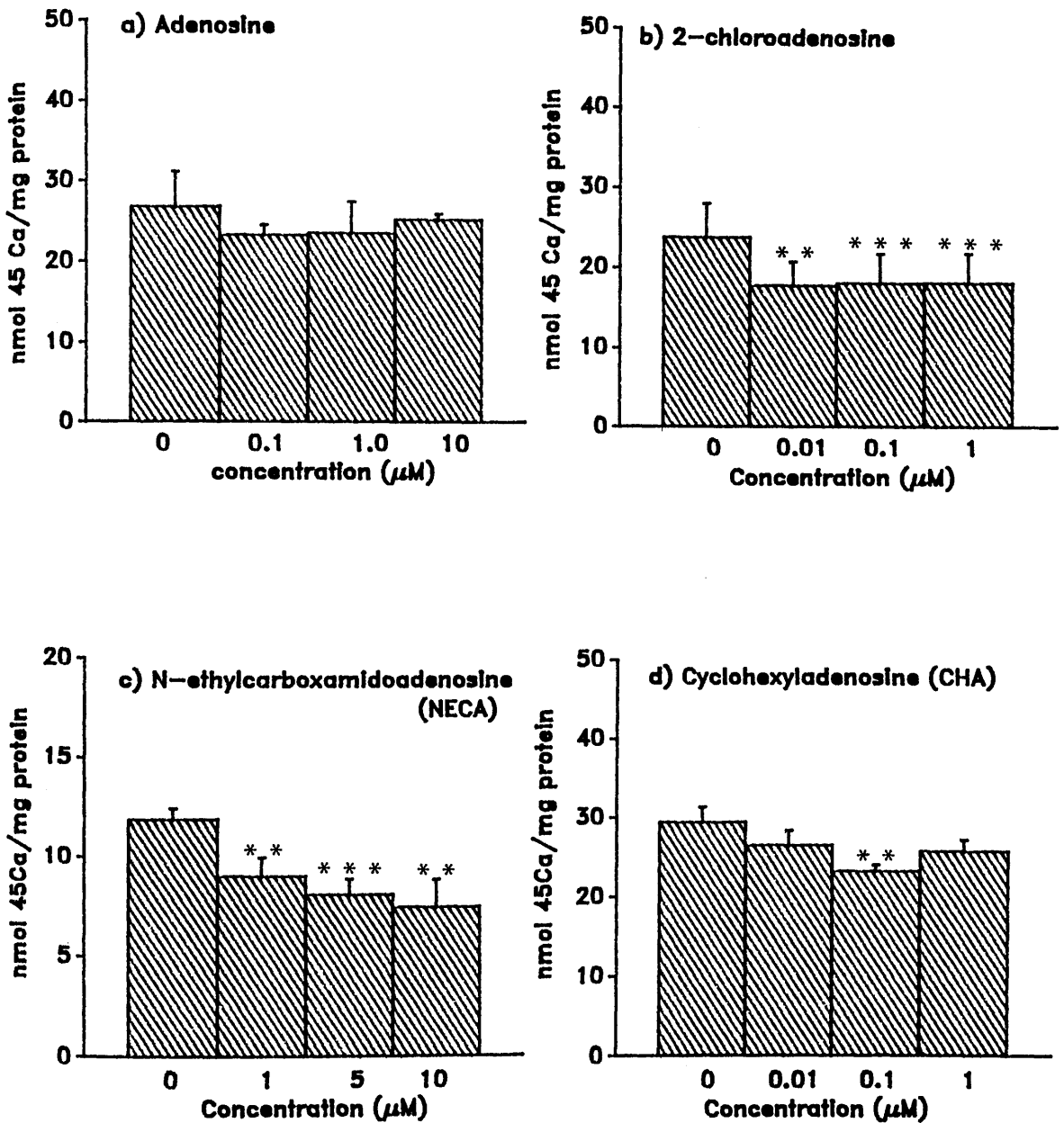
Fig. 3.6. The effect of adenosine compounds on synaptosomal calcium uptake measured over 1 second.



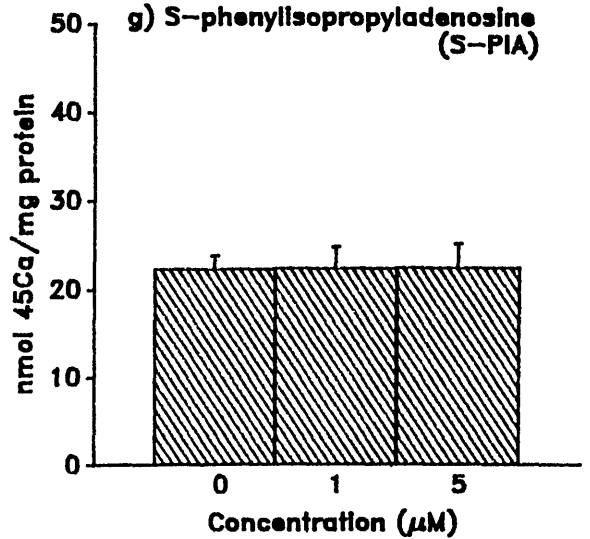
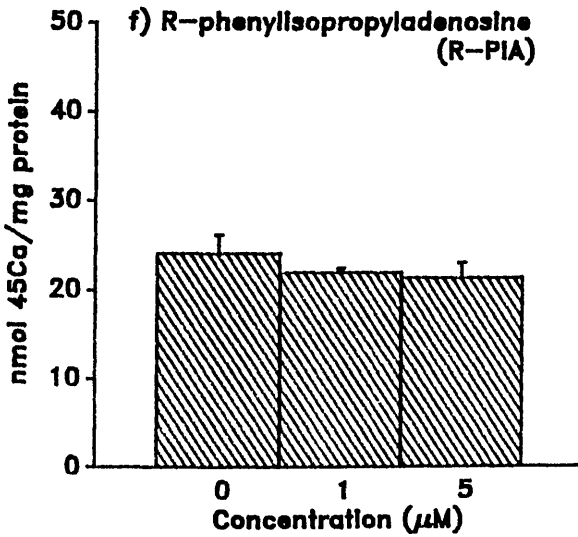
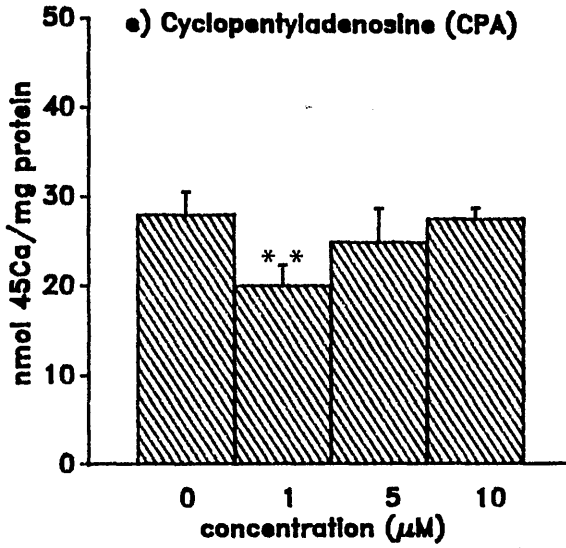
Figs. 3.6.a-d. represent the effect of adenosine compounds on calcium uptake measured over 1 second. Synaptosomes were preincubated for 10 minutes with the compounds at  $37^{\circ}\text{C}$ . Bars represent the mean  $\pm$  sem of 6 determinations. No significant decrease was observed. ( $P > 0.1$ , paired t-test).



Fig. 3.7. The effect of adenosine compounds on synaptosomal calcium uptake measured over 20 seconds.

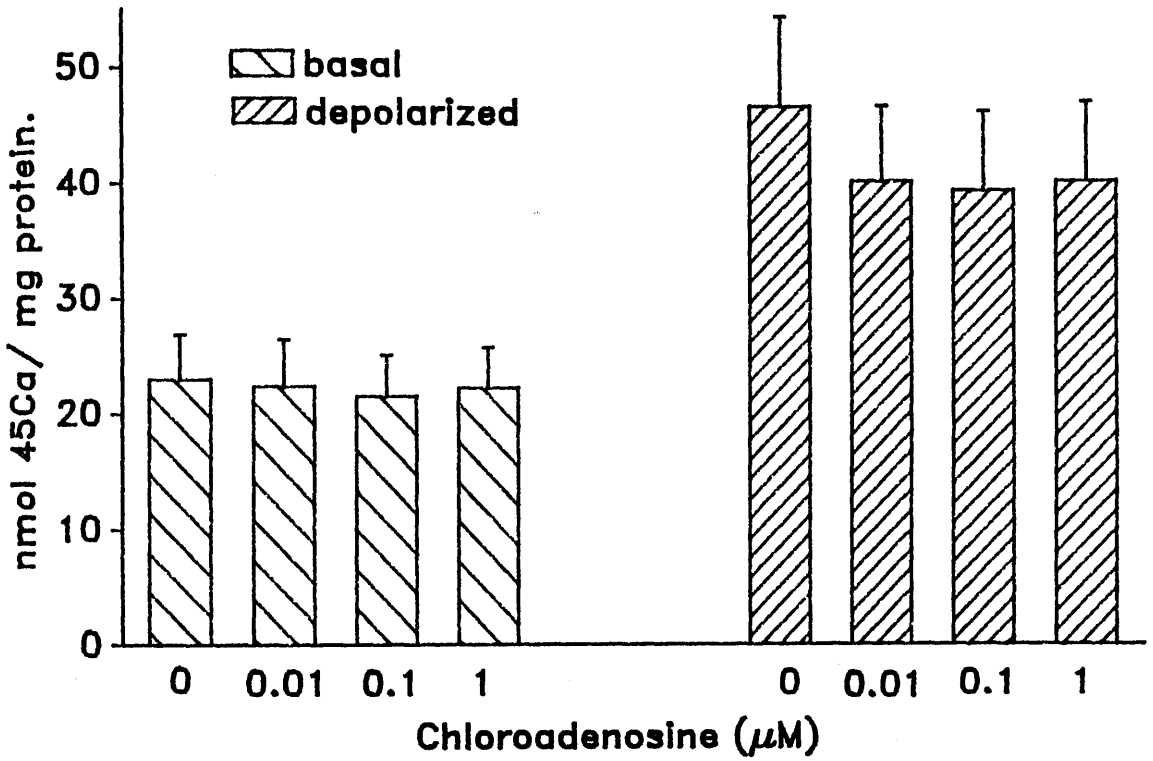


Figs.3.7 a-d represent the effect of adenosine compounds on calcium uptake measured over 20 seconds. Synaptosomes were preincubated for 10 minutes with the compounds at 37°C. Bars represent the mean  $\pm$  sem of 6 determinations. A significant decrease is shown by the asterisks. (\*\* =  $P < 0.05$ , \*\*\* =  $P < 0.01$ ; paired t-test).



Figs.3.7 e-g represent the effect of adenosine compounds on calcium uptake measured over 20 seconds. Experimental parameters as for figs.3.7 a-d. Bars represent the mean  $\pm$  sem of 6 determinations. (\*\* =  $P < 0.05$ , paired t-test).

Fig. 3.8. The effect of 2-chloroadenosine on basal and depolarized calcium uptake.



Data from fig.3.7b showing the effect of 2-chloroadenosine on calcium uptake into basal and depolarized synaptosomes. Bars represent the mean  $\pm$  sem of 6 determinations.

### 3.3.7. The effects of 8-phenyltheophylline and adenosine deaminase.

It was possible that endogenous adenosine might already be exerting an inhibitory action on calcium uptake and so reducing control levels of calcium uptake or reducing the efficacy of added analogues by reducing their binding. To control for this, studies were carried out using adenosine antagonists to remove the presence, or inhibit the action of, endogenous adenosine.

#### 3.3.7 (i). 8-phenyltheophylline.

8-phenyltheophylline (8PT), a potent adenosine antagonist, was added at varying concentrations and uptake times to block the action of endogenous adenosine. An increase in calcium uptake would indicate the presence and action of any endogenous adenosine (Fig.3.9).

No increase in calcium uptake was observed in any of the experiments, suggesting either that no endogenous adenosine was present or that it was not exerting a tonic inhibition on synaptosomal calcium uptake.

Fig. 3.9. The effect of 8-phenyltheophylline on synaptosomal calcium uptake.

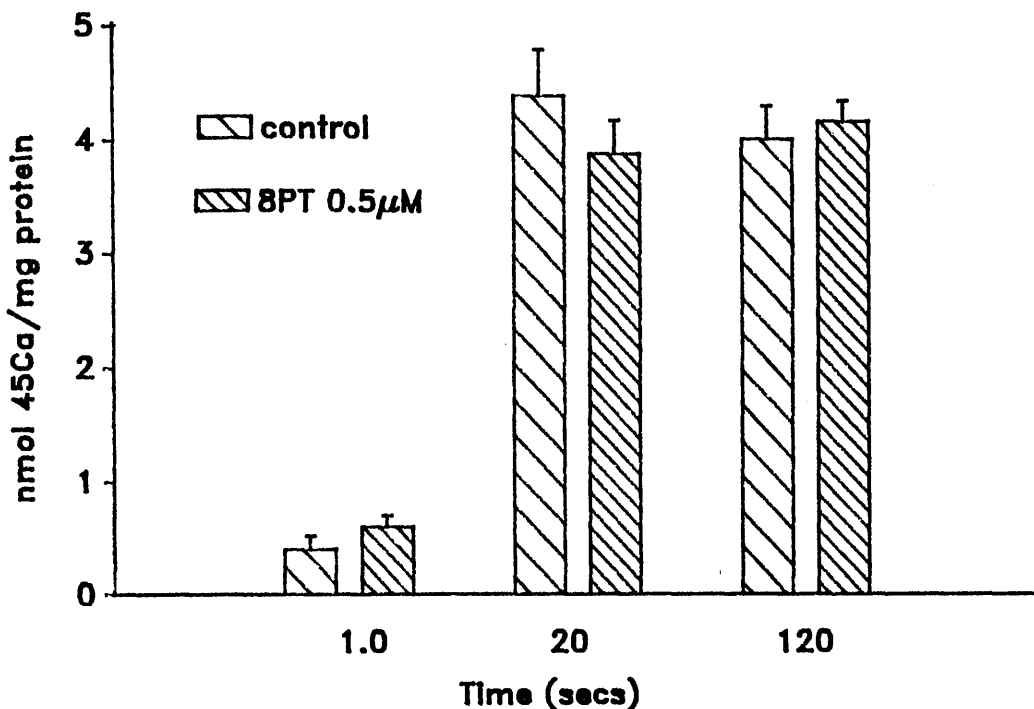
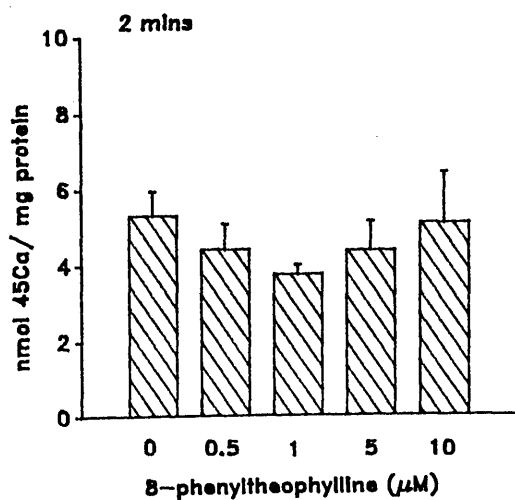
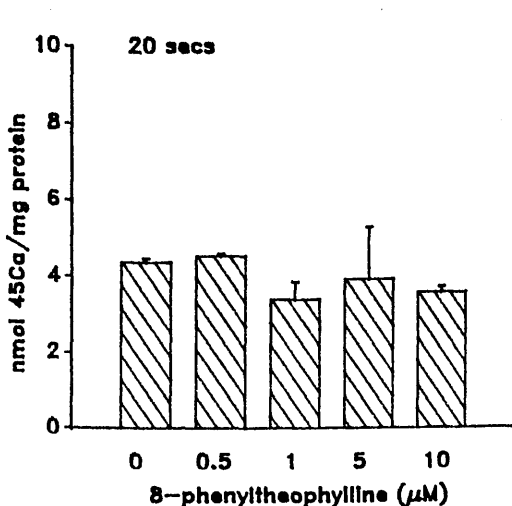


Fig.3.9a. 8PT was preincubated at 37°C with the synaptosomes for 10 mins. Uptake was measured over 1, 20 and 120 seconds. Bars represent the mean  $\pm$  sem of 3 determinations. There was no significant difference between treated and untreated synaptosomes ( $P > 0.1$ , PTT).



Figs. 3.9b & 3.9c. 8PT at increasing concentrations was added directly to the tubes. The synaptosomes were prewarmed for 10 mins at 37°C and uptake was measured over 20 and 120 seconds. Bars represent the mean  $\pm$  sem of 3 determinations. No significant or consistent difference was observed.

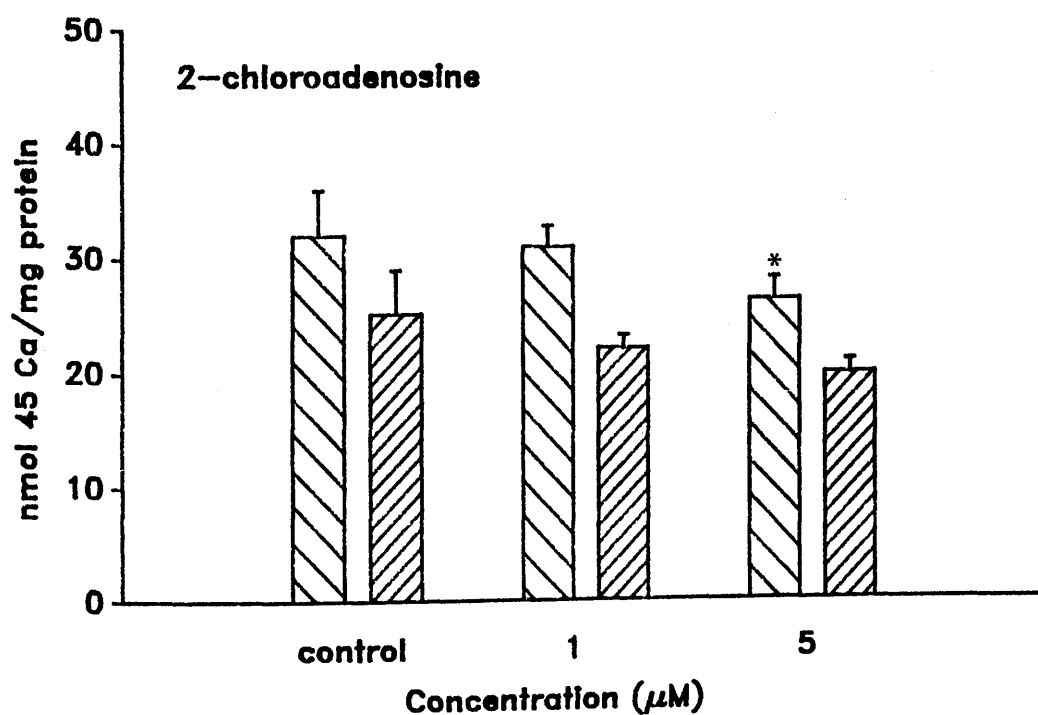
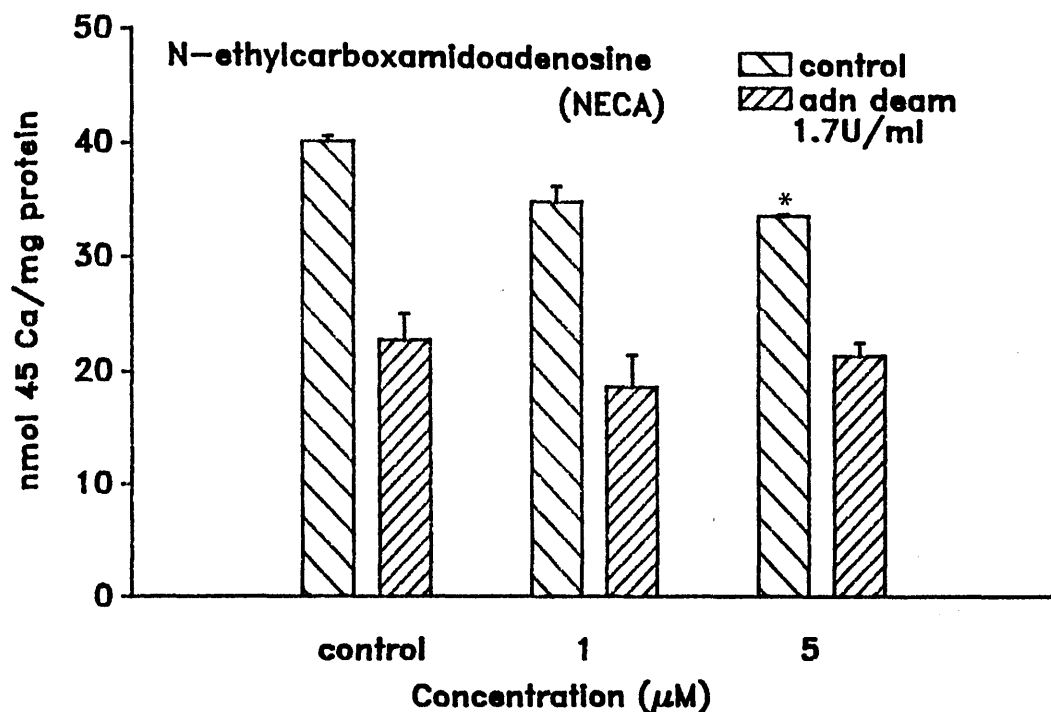
### 3.3.7 (ii). Adenosine deaminase.

Synaptosomes were pretreated with adenosine deaminase (1.7 units/ml, Type II, Sigma) to remove any endogenous adenosine. The effect of 2-chloroadenosine and N-ethylcarboxamidoadenosine (NECA) was assessed (Fig 3.10).

An increase in the percentage inhibition of calcium uptake by these drugs might be expected when endogenous adenosine is removed if it is reducing control levels of calcium uptake or impairing the binding of the analogues.

2-chloroadenosine and NECA significantly inhibited calcium uptake in the absence of adenosine deaminase but the level of inhibition was not improved in the presence of adenosine deaminase and was, in fact, reduced. Adenosine deaminase itself appeared to decrease calcium uptake possibly via a non-specific block of channels by the protein molecule.

Fig. 3.10. The effect of removing endogenous adenosine on the inhibition of synaptosomal calcium uptake by adenosine analogues.



Figs. 3.10a & 3.10b. The synaptosomes were preincubated at  $37^{\circ}\text{C}$  for 30 minutes with adenosine deaminase (1.7 units/ml, Type II, Sigma). For the last 10 minutes the analogues were added. Uptake was measured over 20 seconds. Bars represent the mean  $\pm$  sem of 3 determinations. (\* =  $P < 0.05$ , paired t-test.)

### 3.3.8. Adenosine and magnesium

A study was made of the possibility that the action of magnesium on adenosine inhibition, observed in the hippocampal slices (chapter 1), could also be observed in the synaptosomes. If the hypothesis is valid, removal of magnesium should reduce the inhibition of calcium uptake by adenosine. It would also increase calcium uptake by removing the magnesium block on calcium channels (Hagiwara and Byerly, 1981) so any loss of inhibition by adenosine would be impossible to distinguish from the overall increase in calcium uptake. Instead the magnesium concentration was raised to try and enhance the action of adenosine (Fig.3.11).

The synaptosomes were prepared in a magnesium rich ACSF (4mM) and adenosine and 2-chloroadenosine were preincubated with the synaptosomes at 37°C for 10 minutes prior to experimentation. Uptake was measured over 20 seconds.

The increased magnesium had no significant effect on calcium uptake or the ability of adenosine and 2-chloroadenosine to reduce voltage dependent uptake.



Fig. 3.11. The effect of 4mM magnesium on the inhibition of calcium uptake by adenosine and 2-chloroadenosine.

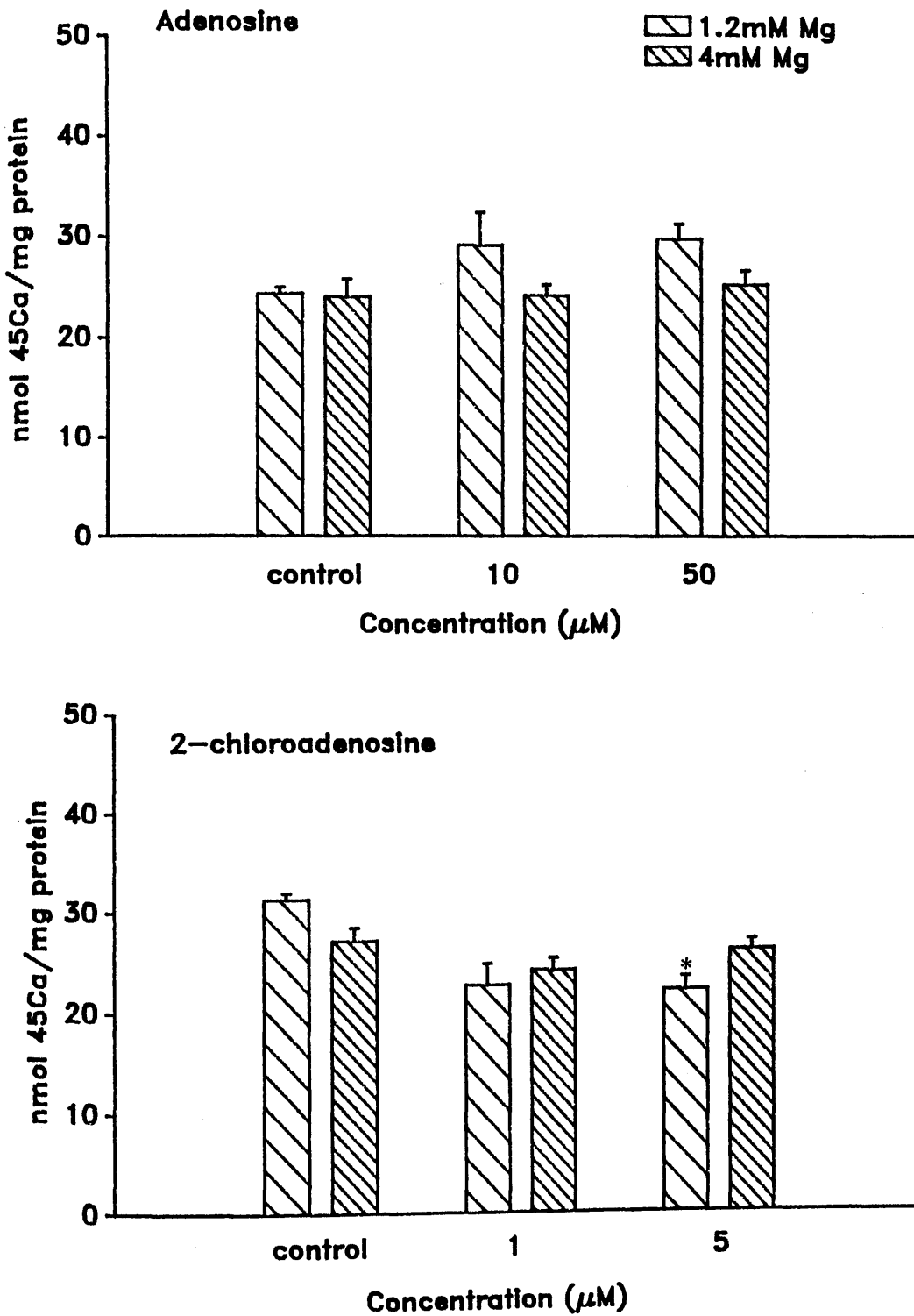


Fig 3.11a & 3.11b. Synaptosomes were resuspended and preincubated with adenosine or 2-chloroadenosine in magnesium rich ACSF (4mM) for 10 minutes at 37°C. Uptake was determined over 20 secs. Bars represent the mean  $\pm$  sem of 3 determinations. (\* =  $P < 0.05$ , PTT).

### 3.3.9. The action of adenosine analogues on calcium uptake into hippocampal slices.

To determine the effect of adenosine on calcium uptake in a more intact tissue preparation, and to tie in with the electrophysiological studies, a series of experiments was carried out using hippocampal slices.

Experiments were carried out to determine the optimal experimental conditions for the slices.

Determination of calcium uptake over time showed stimulated uptake to be greatly enhanced if the slices were prepared and incubated in low calcium (0.12mM) ACSF. Uptake increased over 10 minutes after which it levelled off.

A potassium concentration response curve showed the  $EC_{50}$  for stimulation of calcium uptake to be 50mM. However, to maintain some level of comparison with the synaptosomal experiments, a potassium concentration of 70mM was used to depolarize the slices.

The effect of the adenosine compounds on calcium uptake into the hippocampal slices was determined. Uptake was determined over 10 minutes (Fig.3.13a-e).

Adenosine and 2-chloroadenosine significantly inhibited calcium uptake at high concentrations. CPA significantly inhibited uptake at low concentrations but not at higher concentrations, in a similar manner to that observed with the synaptosomes.

Fig.3.12. Determination of the optimum parameters for the study of calcium uptake into hippocampal slices.

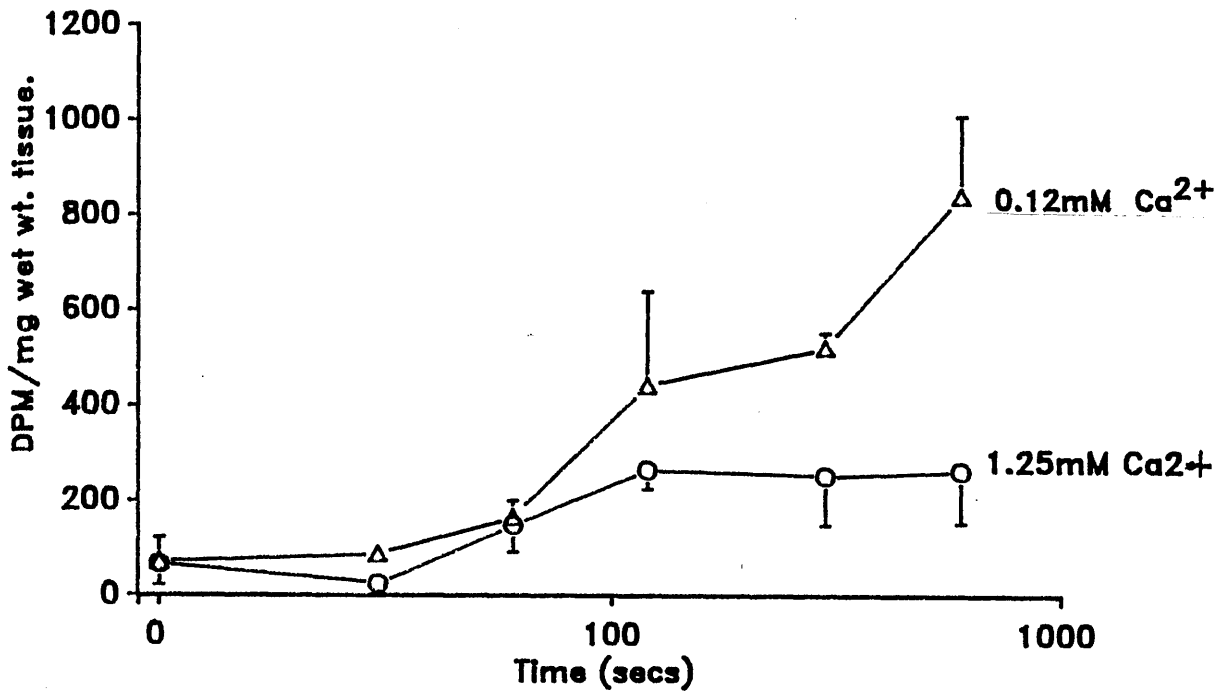


Fig. 3.12a. Calcium uptake into hippocampal slices over time in the presence of normal or low (0.2mM) extracellular calcium. The slices were depolarized using 70mM potassium. Points are mean  $\pm$  sem of 6 determinations.

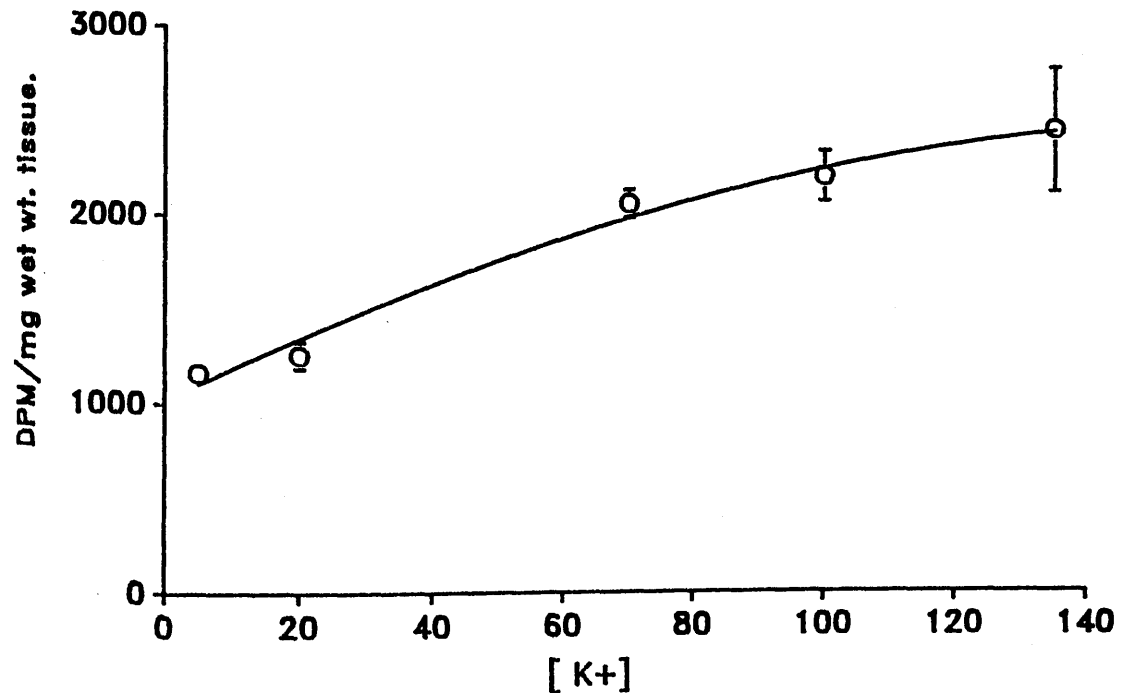
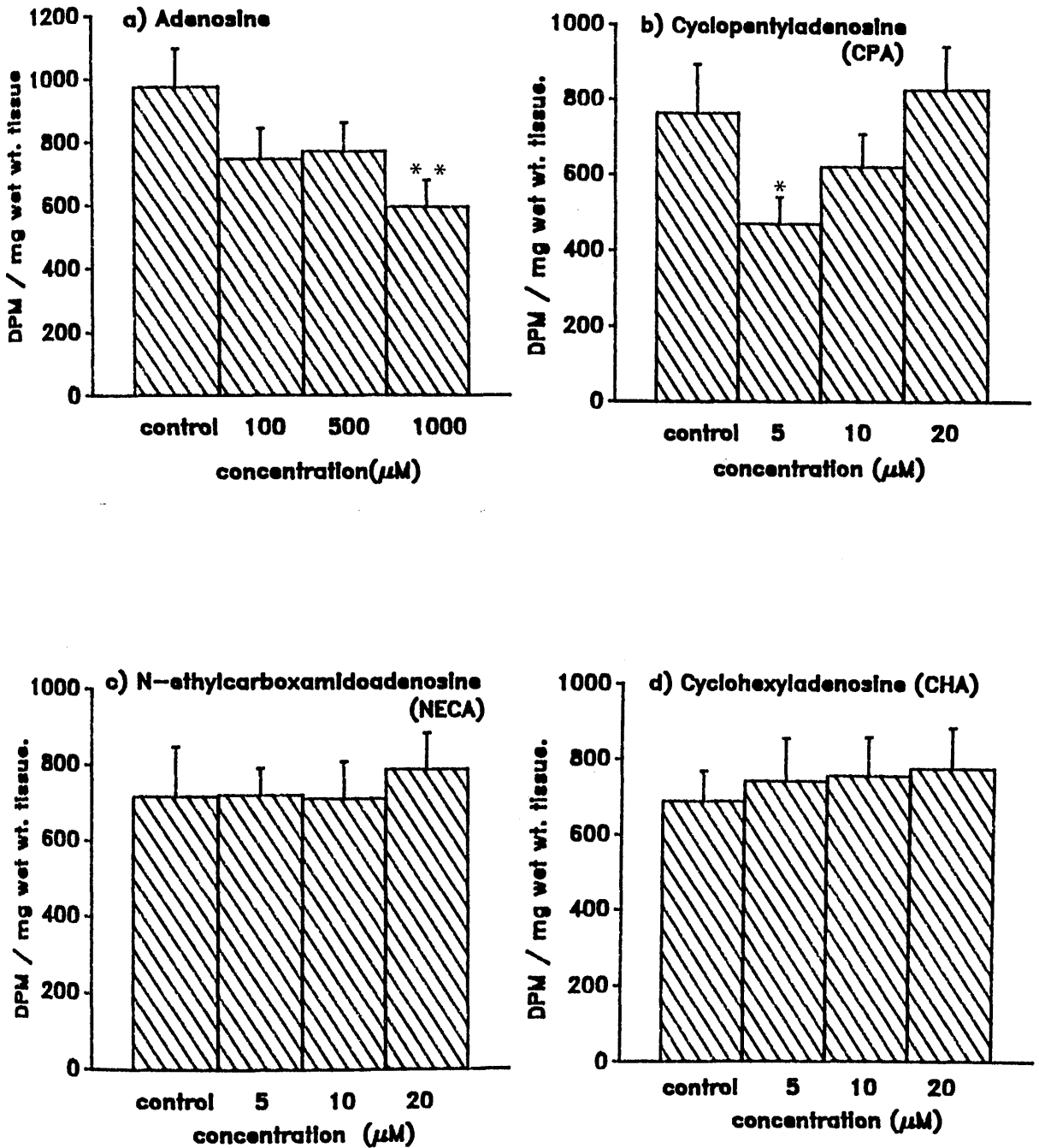


Fig. 3.12b. Calcium uptake into hippocampal slices with increasing potassium concentrations. Uptake was measured over 10 minutes. Slices were prepared and preincubated in low calcium (0.12mM) ACSF. Points are mean  $\pm$  sem of 6 determinations.

Fig. 3.13. The effect of adenosine compounds on potassium stimulated calcium uptake into hippocampal slices.



Figs.3.13 a-d. Effect of adenosine compounds on calcium uptake into rat brain hippocampal slices. 70mM  $\text{K}^+$ , 10 mins uptake. Mean  $\pm$  sem of 12-18 determinations. Drugs were preincubated with the slices for 10 minutes at 37°C. (\* =  $P < 0.1$ , \*\* =  $P < 0.05$ ; paired t-test).

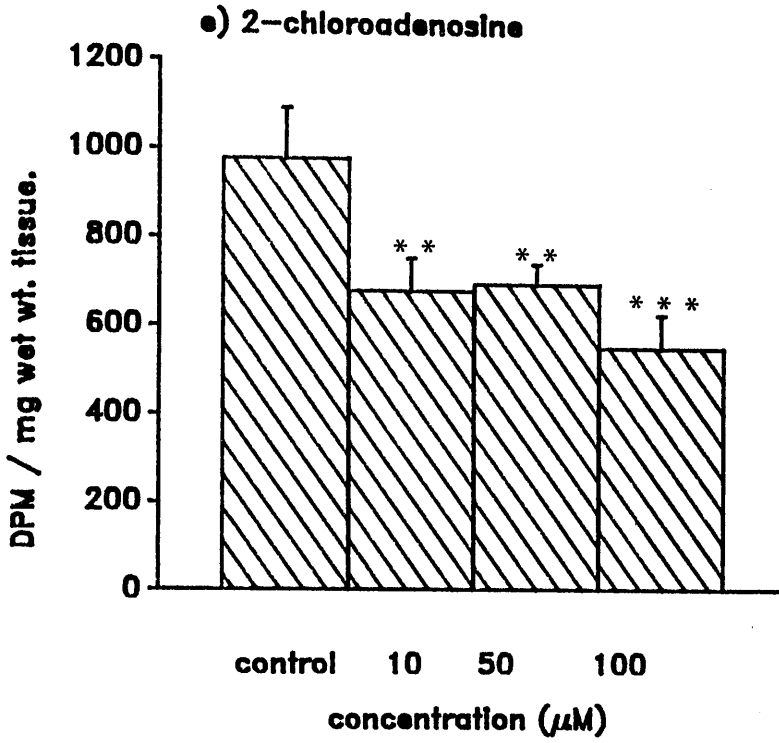


Fig.3.13e. Effect of 2-chloroadenosine on calcium uptake into rat brain hippocampal slices. 70mM  $\text{K}^+$ , 10 mins uptake. Mean  $\pm$  sem of 18 determinations. 2-chloroadenosine was preincubated with the slices for 10 minutes at 37°C. (\*\* =  $P < 0.05$ , \*\*\* =  $P < 0.01$ ; paired t-test).

To confirm that the effects of the adenosine compounds were being exerted via a receptor mediated action, 8-phenyltheophylline was added to antagonize the action of 2-chloroadenosine (Fig.3.14).

8PT totally blocked the inhibition of calcium uptake by 2-chloroadenosine in a dose dependent manner. 8PT on its own had no significant effect on calcium uptake.

Fig. 3.14. Antagonism by 8-phenyltheophylline of the action of 2-chloroadenosine on hippocampal slice calcium uptake.

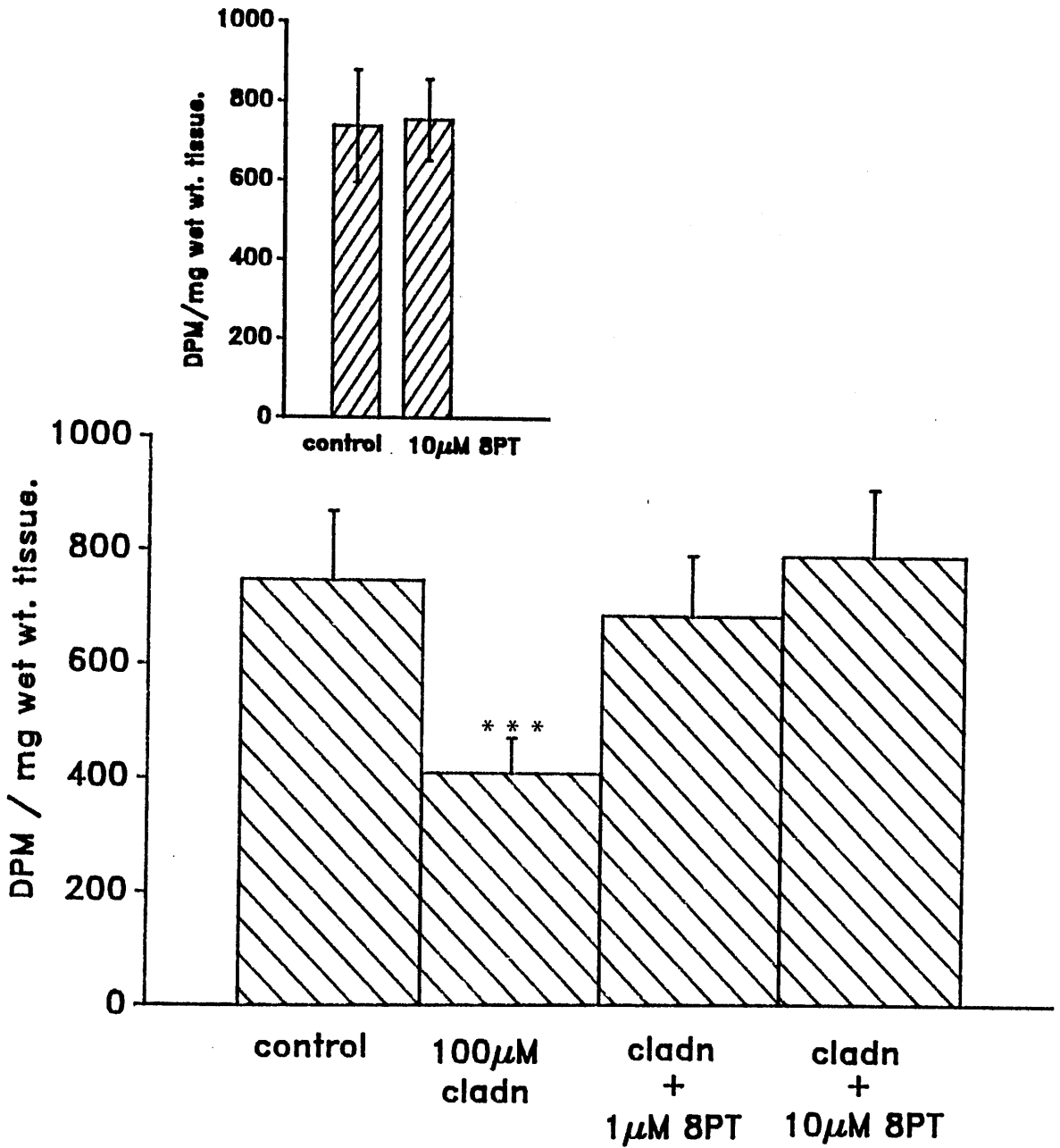


Fig.3.14. The inhibition of calcium uptake into hippocampal slices by 2-chloroadenosine in the absence and presence of 8-phenyltheophylline. Bars represent the mean  $\pm$  sem of 6 determinations (\*\*\*) =  $P < 0.01$ ; paired t-test).

(inset) The action of 8PT on its own had no significant effect on calcium uptake,  $n=3$ .

### 3.4. DISCUSSION

The reduction in calcium influx across voltage sensitive calcium channels by adenosine analogues is small ( $\leq 32\%$ ) but significant. This can be demonstrated in both synaptosomes, representing presynaptic nerve terminals; and in hippocampal slices, representing a more intact region of brain tissue known to be rich in adenosine receptors (Fastbom et al., 1987). However, inhibition of calcium influx was not shown by all the adenosine analogues and the agonist profile was different between the synaptosomes and the slices. The possible reasons for this, and whether the results show a real action of adenosine on neuronal calcium channels, are discussed below.

As the whole rat brain, minus cerebellum, is used to prepare the synaptosomes, the resulting preparation contains a heterogeneous population of nerve terminals. However, the properties of voltage dependent calcium channels on nerve terminals are thought to be consistent throughout the brain (Suszkiw et al., 1986). Also, although adenosine receptors are not distributed evenly throughout the brain (Fastbom et al., 1987), the inhibitory action of adenosine has been demonstrated on most, if not all, neurotransmitters in the brain. Consequently, adenosine should have the potential to act on the majority of synaptosomes in the preparation.

The synaptosomal preparation used in these studies was purified on a sucrose density gradient to reduce contamination from free mitochondria, a potential source of



calcium sequestration. Scott et al. (1980) showed that 79% of the calcium taken up by synaptosomes is sequestered by the intracellular mitochondria, while Adron Harris (1984) found that 97% of the glutamate stimulated calcium uptake in a crude mitochondrial ( $P_2$ ) fraction could be attributed to mitochondrial sequestration. This was reduced to 65% in a purified synaptosomal fraction and was attributed to mitochondria within the synaptosomes. Wu et al. (1982), who used a crude  $P_2$  fraction to study the effect of adenosine on potassium stimulated calcium influx, showed that high potassium produced very little stimulation of calcium uptake in a mitochondrial fraction. So, if only the stimulated calcium uptake is measured, this can be attributed to uptake predominantly into synaptosomes. A purified preparation is still preferable to reduce sequestration of calcium by free mitochondria, leaving more available for synaptosomal uptake.

The glial cells are another possible contaminant of the synaptosomal fraction. These are capable of taking up calcium and adenosine (Hertz et al., 1989; Lewin and Bleck, 1979). Studies by Barnes and Mandel (1981), using cultured glial cells from chick brain, showed basal  $^{45}\text{Ca}$  uptake of only 20% of that seen in cultured neurones and this uptake was not increased by potassium depolarization (60mM). A similar lack of potassium stimulated calcium influx was observed in mouse cultured astrocytes (Walz and Wilson, 1986). In contrast to these studies, Hertz et al. (1989) were

able to show a stimulation of  $^{45}\text{Ca}$  uptake into cultured mouse astrocytes by 50mM potassium. This was achieved only if the  $^{45}\text{Ca}$  was added a minute before the potassium. In the present experiments the synaptosomes were added directly to the stimulating solution and were in contact with the  $^{45}\text{Ca}$  for only as long as the incubation period (20 secs). This should have limited the potential uptake of calcium into glial cells. Even if a percentage of the stimulated uptake was into glial cells, there is no evidence that adenosine can act on glial cells to reduce calcium uptake.

Synaptosomes are metabolically active and able to synthesize ATP using glucose as substrate (Bradford, 1969). This can be metabolized to adenosine that may impose a tonic depression on calcium influx (Silinsky, 1986a) or, alternatively, affect the binding of added analogues (Williams et al., 1986). If a tonic inhibition by endogenous adenosine is present, then addition of 8-phenyltheophylline (8PT) should block this effect, showing up as an increase in calcium influx. If endogenous adenosine is preventing binding, the addition of adenosine deaminase should break down the adenosine and allow the added analogues to exert a maximal effect. No effect on calcium influx was seen using 8PT, while adenosine deaminase actually decreased influx, possibly by a non specific effect. Similar experiments and results were obtained by Garritsen et al. (1989). However, this group also showed that binding of [ $^3\text{H}$ ] DPCPX, an  $\text{A}_1$  receptor antagonist, was increased in the presence of

adenosine deaminase indicating that endogenous adenosine is reducing binding to  $A_1$  receptors. Also, R-PIA was able to displace [ $^3H$ ] DPCPX, indicating that adenosine analogues are able to bind to  $A_1$  receptors in the absence of endogenous adenosine. What was not clear was the effect of R-PIA binding in the presence of adenosine. However, the fact that removing endogenous adenosine did not increase the effect of the analogue suggests that endogenous adenosine is not able to exert any significant effect on depolarized calcium influx.

Following the observation that the action of adenosine was enhanced by increasing the extracellular magnesium ion concentration (Fig.1.4) in electrophysiological studies (chapter 1), a short study was carried out to determine if the extracellular magnesium concentration had any effect on the action of adenosine on synaptosomal calcium influx. An increase in extracellular magnesium might be expected to reduce calcium influx by blocking calcium channels. However, the current experiments did not show a significant reduction in calcium influx in high magnesium (4mM), nor was there any increase in inhibition by adenosine or 2-chloroadenosine. Unfortunately, the synaptosomal preparation does not readily lend itself to the study of a specific interaction of magnesium with adenosine due to the calcium channel regulatory nature of magnesium.

A number of factors have to be considered concerning calcium influx over time periods longer than 1 second and the use of potassium to depolarize the synaptosomes.

It is possible that the inhibition of  $^{45}\text{Ca}$  influx by adenosine observed in these experiments is being partially masked by the use of potassium to depolarize the synaptosomes. Studies by Shinozuko et al. (1985), using synaptosomes prepared from guinea pig ileum myenteric plexus, showed a depression of  $\text{K}^+$  (40mM) stimulated  $^{45}\text{Ca}$  influx by adenosine (100 $\mu\text{M}$ ) of 58%. However, when electrical stimulation was used, adenosine completely abolished the uptake. This effect was also observed by Pinto et al. (1989) in rat cortical synaptosomes.

Electrical stimulation, even at high frequency, may activate calcium channels without allowing them to reach a fully inactivated state so that they are constantly opening and closing over the long (2 mins) period of stimulation. This is in contrast to potassium evoked depolarization where the membrane potential is permanently altered and channels open and close only once in the first second and are then inactivated, possibly by the increased intracellular calcium levels (Suszkiew et al., 1989).

Another piece of experimental evidence reported by Fredholm and Dunwiddie (1988) may also be appropriate to this argument. They studied the phosphorylation of synapsin I, a protein that is phosphorylated in the presence of calcium, the resulting phosphorylated state being

important in the control of transmitter release. Addition of cyclohexyladenosine (CHA) reduced the calcium dependent phosphorylation of synapsin I induced by brief bursts of depolarization by potassium, but the CHA was ineffective on phosphorylation induced by sustained potassium depolarization. This fits with the idea that channels activated by brief depolarizations, such as electrical stimulation, can be blocked by adenosine, while potassium stimulated depolarization is so intense it partially overcomes the inhibitory action of adenosine.

A second possibility is that sodium/calcium ( $\text{Na}^+/\text{Ca}^{2+}$ ) exchange is partly responsible for the late, slow phase of calcium influx observed after the first couple of seconds. This is supported by a number of reports (Coutinho et al., 1984; Turner and Goldin, 1985; Suszkiew et al., 1986) as well as the present study (fig.3.5). This presents two possibilities:

- 1) The potency of adenosine on VSCC influx is being partially masked by adenosine insensitive  $\text{Na}^+/\text{Ca}^{2+}$  exchange calcium influx. This idea is also supported by the electrical stimulation experiments discussed above as electrical stimulation would not affect  $\text{Na}^+/\text{Ca}^{2+}$  exchange.

- 2)  $\text{Na}^+/\text{Ca}^{2+}$  exchange is responsible for all the late phase calcium influx and it is this which adenosine is acting upon. To do this adenosine would need to inhibit the reversal of  $\text{Na}^+/\text{Ca}^{2+}$  exchange, reducing calcium entry via this route. If this was the case then a similar block would

be expected on the basal uptake resulting in an increase in basal calcium levels due to a drop in efflux. This was not observed. A similar argument could be applied if adenosine actively promoted calcium efflux, as has been suggested for adenosine action on the guinea pig taenia-coli (Frischnecht and Ferrero, 1984). There was no significant reduction of basal calcium influx (Fig.3.8).

#### 3.4.1. Fast and slow calcium channels.

Calcium influx into nerve terminals occurs in two phases with an initial rapid phase followed by a late, slow phase (Gripenberg et al.,1980). The initial rapid phase possibly corresponds to influx across the N type channel (Nowosky et al.,1985) that predominate on nerve terminals (Reynolds et al.,1987). This rapid and transient influx is probably responsible for neurotransmitter release in response to synaptic depolarization. If adenosine acts on calcium channels, it would presumably act on this rapid phase to reduce neurotransmitter release.

The late slow phase of calcium uptake may be related to influx across the L type calcium channels. As adenosine appears to show an effect predominantly on longer uptake periods (20 to 120 secs), it may be acting on this channel to reduce presynaptic calcium uptake. However, an effect of adenosine on this channel is not consistent with an immediate action on neurotransmitter release. Also, the dihydropyridines are thought to act on the L channel but have little effect on synaptic transmission.

A third possibility is that of a distinct calcium current in nerve terminals as suggested by Suszkiew et al. (1989). This potassium depolarization dependent calcium channel ( $J_{Ca}$ ) was first identified in Xenopus oocytes injected with rat brain mRNA (Leonard et al., 1987). The kinetic properties are distinct from the other three established calcium channels (T, N and L); it activates at moderate ( -50mv) depolarizations, inactivates slowly (0.8 sec) and is insensitive to dihydropyridines and w-conotoxin. The inactivation time can be increased to almost 10 secs if depolarized with low (25mM) potassium and low calcium medium, calcium being thought to decrease the inactivation time.  $J_{Ca}$  is also much more sensitive to block by cadmium as compared with cobalt.

If  $J_{Ca}$  is the predominant voltage activated calcium channel in rat brain synaptosomes then it is possible that adenosine is acting on this channel. Maximum influx is seen at around 20 seconds. Assuming the calcium channels are not all activated at the same time then a channel exhibiting a slow inactivation rate, the population of which takes a number of seconds to reach full inactivation, would fit with the data presented in this chapter and in a number of other publications (Ribeiro et al., 1979; Wu et al., 1982). The membrane potential of synaptosomes prepared by the technique used for the present experiments was calculated by the Pfizer scientists to be -73mv at rest, rising to -38mv upon depolarization with 70mM  $K^+$ . This further supports the

existence of a distinct synaptosomal calcium channel activated at low membrane potentials.

The fact that adenosine effects could not be observed at shorter (1 sec) uptake times, in this or other studies (Barr et al., 1984; Garritsen et al., 1989), may be due to the fact that maximum uptake is not reached in this short period, and the relatively small percentage decrease in calcium influx by adenosine can only be shown to be significant after maximum influx has occurred. Alternatively, there may be insufficient mixing of the synaptosomes with the potassium, calcium and adenosine compounds during this brief period, preventing maximum activation of the calcium channels. The apparatus used by Suszkiew et al. (1986) involved instantaneous, electronically controlled mixing that allowed measurement of uptake down to 50 msec. It would be interesting to see the effect of adenosine on rapid calcium fluxes using this system.

#### 3.4.2. Adenosine Receptors.

The agonist profile determined in this study does not fit either the A<sub>1</sub> or the A<sub>2</sub> established profiles, so it is not clear what type of adenosine receptor on the synaptosomes is being acted upon by the adenosine analogues. It is possible that the action of CHA and R-PIA was impaired by the vehicle used to dissolve them (see below), in which case the analogues may prove to be acting at an A<sub>1</sub> receptor. However, there are other possibilities.



Ribeiro and Sebastião (1986) have proposed a third  $A_3$  adenosine receptor that is not coupled to adenylate cyclase but instead regulates calcium flux. This could be either via direct action; the receptor linked to, or part of, a voltage sensitive calcium channel; or indirectly, the activation of the receptor leading to a conformational change in the membrane that alters VSCC activity. This proposed receptor would be found on nerve terminals and heart tissue, as calcium influx is a major component of cell excitability in these tissues. The basis for proposing a third receptor is the continual observation of agonist profiles that fit neither  $A_1$  nor  $A_2$  established profiles, particularly in studies on heart and neuronal tissue (e.g. Jonzon and Fredholm, 1984; Hughes and Stone, 1983). In particular, functional studies on neuronal preparations rarely show agonist profiles that suggest an  $A_2$  receptor, while functional effects of adenosine, designated as acting via  $A_1$  receptors, are based upon the stereoselectivity of the PIA isomers rather than the agonist profile as a whole. Ribeiro and Sebastiao (1986) suggest that the  $A_3$  receptor can account for those profiles that do not neatly fit an  $A_1$  receptor profile. The  $A_3$  profile is CHA, R-PIA, NECA equipotent, with 2-chloroadenosine being less potent. Unfortunately, the adenosine receptor that is being acted upon in my study does not fit the profile of an  $A_3$  receptor as stipulated by Ribeiro and Sebastiao (1986). 2-chloroadenosine is as potent as NECA, and R-PIA has no effect at the concentrations used. This is also the case in

the study by Wu et al. (1982) where 2-chloroadenosine was the most potent.

Another claim for a new  $A_3$  adenosine receptor linked to calcium systems in the brain has been put forward by Chin and Delorenzo (1986). Using 2-chloro[ $^3H$ ]adenosine (Cl[ $^3H$ ]adn) as a ligand they demonstrated an adenosine binding site in cortical membranes with a micromolar affinity. Cl[ $^3H$ ]adn binding to this receptor was unaffected by R-PIA, 8PT or NEM treatment. Pretreatment of the tissue with adenosine deaminase exposed a high affinity receptor with preference for R-PIA which was the  $A_1$  receptor. Untreated tissue did not show the  $A_1$  receptor, only the "novel" receptor. The order of potency for displacement of Cl[ $^3H$ ]adn was 2-chloroadenosine > NECA > R-PIA. 8PT had no effect on binding up to 10  $\mu M$ . This also differentiated it from the  $A_2$  receptor. This " $A_3$ " receptor was found to be particularly rich in hippocampal membranes (Chin et al., 1985). Cl[ $^3H$ ]adn binding was also stimulated by the addition of cobalt, nickel and lanthanum, this stimulation being antagonized by calcium (Chin and Delorenzo, 1985). The agonist profile for this receptor fits the profile determined in the present study, so it is possible that a receptor, similar to the one proposed by Chin and Delorenzo (1986), is responsible for the effect of the adenosine receptor agonists on calcium influx observed in the present study. Unfortunately the characterization of this receptor is based entirely on Cl[ $^3H$ ]adn ligand binding studies with no functionally

correlated studies to support it. However, there is room for a third adenosine receptor to explain a number of receptor studies that do not fit either of the established  $A_1$  or  $A_2$  receptor classes. It is possible that the action of adenosine on presynaptic calcium channels will be one of the actions of this theoretical receptor.

### 3.4.3. Calcium uptake into hippocampal slices.

Brain slices have been used extensively to study the action of the excitatory amino acids on calcium influx (Ichida et al., 1982; Retz and Coyle, 1984; Crowder et al., 1987), but this has not been extended to studies on adenosine. It was therefore decided to see if potassium stimulated calcium influx into hippocampal slices could be affected by adenosine.

Trial studies were carried out with the slices to determine if electrically stimulated calcium uptake could be measured. However, it proved difficult to find suitable conditions under which a significant stimulation of calcium uptake could be observed, so this approach was abandoned due to lack of time.

In a slice of brain tissue there is no way to distinguish exactly which cells in the slice are taking up calcium or being affected by adenosine. Glial cells may contribute to the observed calcium uptake. As discussed earlier,  $^{45}\text{Ca}$  potassium stimulated influx into cultured astrocytes has been observed (Hertz et al., 1989) and the 10 minute

incubation time for the hippocampal slices may have allowed a percentage of the  $^{45}\text{Ca}$  to be taken up into glial cells. However, a study by Walz and MacVicar (1988) found that astrocytes in hippocampal slices did not express calcium channels, unlike cultured astrocytes. The reason for this is unclear, although poor energy metabolism in the slices may have inactivated the astrocyte calcium channels rendering them undetectable. Whether the channels activate under potassium stimulation in the slice is also unclear, but again there is no evidence that adenosine acts on glial cell calcium influx, and the observed inhibition of calcium influx by adenosine is likely to have been into neurones rather than glial cells.

In the hippocampal slice experiments the adenosine analogues had a different order of potency from the synaptosomal studies. Adenosine itself produced a dose dependent decrease in calcium influx that was not observed in the synaptosomes. 2-chloroadenosine was effective in both studies, while NECA appeared to have no effect on hippocampal calcium influx. An obvious difference between the two studies was the greatly increased concentrations of drugs used in the slices. As the slice is a relatively intact piece of tissue, the drugs need to pass into the tissue before they can exert a maximal effect on as many receptors as possible. The actual concentration of drug around the receptor sites may be much lower than the added concentration. A second reason, as discussed by

Dunwiddie (1985) and one that is also applicable to the synaptosomes, is that the concentration of potassium may reduce the observed inhibition so that higher concentrations of drug are needed to show a significant reduction.

A second, interesting comparison between the two studies is the action of cyclopentyladenosine (CPA). In both studies, CPA showed a decrease in calcium influx at low concentrations but not at higher concentrations. The reason for this is unclear. One possibility is that the vehicle used, ethanol, antagonized the effect of the agonist at higher concentrations, although the maximal concentration of ethanol was never more than 0.5%. CHA was also dissolved in ethanol and this also exhibited a small inhibition on synaptosomal calcium influx that was not dose dependent. S and R-PIA were dissolved in DMSO (max final concentration <0.5%) and it is possible that this vehicle also prevented the action of the analogues. However, although it is not made clear in the publications, there is no reason to believe that other studies (e.g. Wu et al., 1982) were able to dissolve these drugs without using the usual vehicle.

In conclusion, the adenosine analogues appear to be able to exert a partial inhibition on calcium influx. This may not be representative of the true ability of adenosine to block calcium channels, as the use of potassium to depolarize the synaptosomes may be attenuating the action of adenosine. There may also be a component of calcium influx

that occurs via the  $\text{Na}^+/\text{Ca}^{2+}$  exchange which is unlikely to be sensitive to adenosine and will reduce the true level of inhibition by adenosine. Adenosine itself was ineffective on synaptosomal calcium influx, and active only at high concentrations in the slices. This may have been due to rapid metabolism or uptake of adenosine. Optimal parameters, under which the true effect of adenosine on calcium channels in synaptosomes could be ascertained, would be to use electrical depolarization, a short uptake time (1-5 secs) and the addition of an adenosine uptake inhibitor to maximise the effect of adenosine.

## GENERAL DISCUSSION

## GENERAL DISCUSSION

The experiments reported in this thesis have attempted to look at the effect of adenosine on calcium associated events in the rat brain using electrophysiological and neurochemical techniques. This has revealed some interesting interactions between adenosine and other calcium associated mechanisms, namely the NMDA receptor operated channel and the dihydropyridine calcium channel antagonists. The author's conclusions about the possible actions occurring in the experiments are summarized below and the relevance of these effects to the role of adenosine in the CNS are discussed.

The results from chapter 1 show a reduction in adenosine inhibition in magnesium free ACSF that appears to be the result of increased NMDA receptor activation. When NMDA channels are activated, either by removing extracellular magnesium or by increasing cell excitability with 8.5mM potassium, the inhibitory action of adenosine is greatly reduced. In magnesium free conditions the maximum level of inhibition by adenosine is also reduced so that, in some slices, 1mM adenosine failed to completely abolish synaptic transmission.

Other reports of reduced inhibitory action by a lack of magnesium in functional studies are limited, but there is evidence to support the observations of the present experiments.



Artemenko and Gerasimov (1984) looked at the effect of magnesium and calcium ions on inhibition of synaptic transmission by adenosine in the rat hippocampal slice. An increase in the magnesium ion concentration increased the inhibitory action of adenosine on the CA1 EPSP, while a decrease reduced it. Changes in the calcium ion concentration had the opposite effect. The  $IC_{50}$  value for adenosine was between 10 and 25  $\mu$ M. When adenosine was tested in ACSF containing low magnesium (0.02mM) and high calcium (4mM) the  $IC_{50}$  for adenosine increased to 90  $\mu$ M, while the maximum inhibition by adenosine was only 65% even at 1mM adenosine. This was taken to indicate that the increased calcium concentration was allosterically inhibiting the binding of adenosine to its receptor, or that calcium was competing with adenosine to occupy a site on a calcium channel. The possibility that a lack of magnesium ions was responsible for the reduced adenosine effect was not seriously considered due to a lack of evidence. However, the results of Artemenko and Gerasimov (1984) appear to support the idea of a lack of magnesium ions impairing the action of adenosine, and the increased calcium concentration may be enhancing the effect by increasing calcium influx across the NMDA channel.

A recent report also provides evidence to support a requirement for magnesium in the action of adenosine. Lloyd et al. (1989) looked at the effect of 2-chloroadenosine on the evoked monosynaptic reflex (MSR) in the isolated

neonatal rat spinal cord. The  $IC_{50}$  for depression of the MSR by 2-chloroadenosine in normal ACSF (1.25mM Mg) was 0.41 $\mu$ M. This increased to 3 $\mu$ M when magnesium was omitted. Furthermore, the maximal inhibition of the MSR was reduced from 100% in normal ACSF to only 65% even at 100 $\mu$ M 2-chloroadenosine. A 30% increase in excitability of the cells was observed upon entry into magnesium free ACSF, but the reduction in the maximum obtainable depression was taken to show that the reduced inhibition was not simply a result of the increased excitability. Neither of these reports looked for a possible NMDA mediated effect, but it is possible that a similar mechanism to the one proposed in the present studies is also being exerted in the above studies.

To try to explain the drop in adenosine inhibition with increased NMDA receptor mediated calcium channel action, the possibility of an interaction between the NMDA channel and the adenosine receptor or associated channel has been considered. The evidence for such an interaction is limited and largely conjectural. Perhaps the most interesting report is by Stratton et al.(1988) who observed a loss of adenosine inhibition on the dentate gyrus population potential in magnesium free ACSF. This effect was irreversible upon reintroduction of magnesium and appeared to be mediated by activation of NMDA channels as the effect was blocked if 2-AP5 was added before the magnesium free ACSF or if the slice was not stimulated during the magnesium free period.

An interaction between adenosine and NMDA receptor operated channels may be of relevance to a number of hypothesized actions of NMDA channel activation including long term potentiation, kindling and epileptiform activity.

Long term potentiation (LTP) is the prolonged enhancement of synaptic transmission that follows tetanic afferent stimulation. This occurs most prominently in the hippocampus and is looked upon as a likely mechanism for learning and memory (Collingridge and Bliss, 1987). LTP is thought to involve activation of NMDA receptor channels, as NMDA antagonists readily block the induction of LTP (Harris et al., 1984). NMDA receptors are rich in the CA1 and dentate gyrus regions of the hippocampus (Cotman et al., 1987) where LTP is most readily recorded. Under normal conditions, NMDA receptor operated channels are not activated during synaptic transmission; glutamate acting at kainate and quisqualate receptors only (Collingridge et al., 1983). However, during a rapid burst of stimuli, the magnesium ions that normally block the voltage dependent NMDA receptor channel are removed by the depolarization of the membrane, and calcium ions, which are thought to play a role in activation of LTP, can enter. The calcium dependence of LTP has also been demonstrated as it can be prevented by reducing the extracellular calcium concentration (Dunwiddie and Lynch, 1979) and induced by increasing calcium (Turner et al., 1982).

Modulation of LTP by other neurotransmitters and neuromodulators has been studied. The inhibitory neurotransmitter GABA is thought to regulate the activity of NMDA receptors in LTP. GABA antagonists such as picrotoxin, bicuculline and penicillin have been shown to facilitate the generation of LTP in hippocampal slices (Wigstrom and Gustafsson, 1983). The opiates may have some regulatory action on LTP, as naloxone can inhibit LTP in the CA3 (Stringer et al., 1983), although opioid antagonists do not appear to promote LTP. Noradrenaline also has an effect on LTP, both attenuating and enhancing it (Collingridge, 1985) possibly depending on the receptor subtype affected (Stanton et al., 1987). Neurotransmitters, such as dopamine, 5HT and acetylcholine appear to have little or no effect on LTP (Collingridge, 1985). Thus, it appears that LTP can be regulated by other neurotransmitters and neuromodulators.

It is therefore not inconceivable that adenosine, which can oppose the depolarization necessary to overcome the magnesium block of NMDA channels, might be able to regulate LTP. Alternatively, the opposite interaction may occur whereby activation of NMDA channels is able to suppress the inhibitory action of adenosine. This may be important for LTP if the tonic inhibition imposed by endogenous adenosine (Ault and Wang, 1986) has to be reduced to allow LTP to be initiated and / or maintained. There may also be an increased release of adenosine as a result of the excitatory inputs that trigger LTP, the inhibitory action of which would also need to be reduced to allow LTP to occur.

The reports of an effect of adenosine on LTP are limited, but suggest that adenosine can affect LTP. Dolphin (1983) looked at LTP induced in vivo in the rat dentate gyrus. Application of 2-chloroadenosine, via a cannula placed in the granule cell body layer of the dentate gyrus, blocked the onset of LTP normally observed upon tetanic stimulation of the perforant pathway. 2-chloroadenosine only needed to be present during the tetanic phase to prevent LTP, but was ineffective if added immediately after stimulation. The results were taken to indicate that 2-chloroadenosine could reduce calcium influx either pre- or postsynaptically, calcium influx being a vital component of LTP onset. The fact that 2-chloroadenosine was ineffective in blocking LTP if added after the tetanic stimuli may be due to a reduction in adenosine action as a result of NMDA channel activation. A loss of 2-chloroadenosine action on synaptic transmission after LTP had been triggered was not reported by Dolphin, so adenosine action is not universally reduced, as is the case in the present study when the majority of NMDA channels are activated in magnesium free ACSF. However, a local effect on those synapses responsible for triggering LTP may occur.

A similar report by Mendoca and Ribeiro (1989) found that 2-chloroadenosine inhibited LTP induced in the CA1 region of the hippocampus. As with the previous report, 2-chloroadenosine was only effective if added prior to LTP onset.

In a similar manner, kindling, a method of inducing spontaneous seizures by repeated electrical stimulation, either in vitro (Slater et al.,1985) or in vivo (Goddard et al.,1969), appears to involve an increase in NMDA channel activation. Studies of kindling in both the CA1 (Wadman et al.,1985) and the dentate gyrus (Mody and Heinemann, 1987) have shown an increased excitability of kindled cells as compared to control and this excitability is readily blocked by 2-AP5. Similarly, kindled epilepsy in the amygdala of rats corresponds to a significant increase in glutamate release, the neurotransmitter thought to act at the NMDA receptor, while other amino acids showed no increase in release (Peterson et al.,1983).

Electrical stimulation and epileptiform activity have both been shown to increase adenosine release (Pull and McIlwain, 1972; Winn et al.,1980). This might be expected to counteract the onset of spontaneous activity, while kindled epilepsy has been shown to be regulated by adenosine agonists and antagonists, attenuating and facilitating it respectively (Dragunow et al.,1985). However, if increased NMDA channel activity is responsible for the kindled seizures, in a similar manner to the induction and maintenance of LTP, then this might counteract the effectiveness of the released adenosine, preventing it from totally inhibiting the spontaneous activity.

Finally, the spontaneous epileptiform activity observed in magnesium free ACSF, thought to be exerted via NMDA receptors (Stanton et al., 1987), may also be enhanced by a reduced presynaptic adenosine action. The anticonvulsant properties of adenosine tend to argue against this possibility, but the postsynaptic action of adenosine, possibly at extrasynaptic or even somatic sites (Tetzlaff et al., 1987), may be responsible for the anticonvulsant effects, while a reduced presynaptic adenosine effect initially allows the NMDA mediated action to go ahead.

These experimental models are likely situations where an interaction between NMDA and adenosine could occur. Assuming LTP is a naturally occurring phenomenon, this might be a condition where a depression of adenosine inhibition could be of physiological relevance.

Before the NMDA component of increased synaptic transmission became apparent, the original hypothesis to explain the reduced adenosine inhibition in magnesium free ACSF was a requirement for magnesium ions at or near the adenosine receptor to enable adenosine to bind to its receptor. This hypothesis is supported by ligand binding studies. Although the experimental evidence of the present experiments cannot be used to show a direct interaction between magnesium and adenosine at the adenosine receptor, there is still a rationale for suggesting a requirement for magnesium for effective adenosine action.

A requirement for magnesium for effective regulation of neuronal function might be expected if it is of importance to the action of adenosine. This is found to be the case, with a number of reports demonstrating the importance of magnesium to neuronal function, and variations in magnesium levels being associated with pathological conditions.

The level of total and intracellular free magnesium has been shown to decrease following traumatic brain injury in rats (Vink et al., 1987). Intracellular magnesium levels were measured in vivo using  $^{31}\text{P}$  magnetic resonance spectroscopy. Following injury of the brain by forced injection of a bolus of  $37^\circ\text{C}$  isotonic saline directly into the cranial cavity, the intracellular free magnesium level fell by 70% within the first hour after injury and did not recover over the remaining 3 hours of observation. Total magnesium levels were determined after 4 hours by atomic absorption spectroscopy and were found to be 10% less than control. Treatment of rats with  $\text{MgSO}_4$  ( $125\mu\text{mol}$ ) 30 minutes after brain injury was performed, protected the animals from chronic neuronal damage. Treatment with  $\text{ATP-MgSO}_4$  or  $\text{ATP}$  provided no significant protection (McIntosh et al., 1989). A 70% reduction in intracellular magnesium concentration could explain a number of post-traumatic neurological effects, the majority of these effects ultimately leading to secondary cell death via injury processes that remain unclear. It is known that a wide range of fundamental biochemical processes depend on the presence of magnesium ions for effective



enzymatic action (McIntosh et al., 1989; Cech et al., 1980). It is possible that an effect on adenosine metabolism by a reduction in magnesium levels is one effect that contributes to the ultimate neuronal damage.

An effect of magnesium that may be more closely related to a possible interaction with adenosine is the effect of magnesium on epileptic seizures. Eclampsia, epileptic seizures experienced during pregnancy, have been regularly treated with magnesium sulphate administered intramuscularly or intravenously (Valenzuela and Munson, 1987; Dinsdale, 1988). The pre-eclamptic state is typically characterized by a reduction in plasma magnesium levels together with acute hypertension. Administration of magnesium in the pre-eclamptic state prevents the onset of convulsions, and arrests convulsions if administered after onset. The way in which magnesium acts to inhibit seizures is unclear. A neuromuscular blocking action may be responsible for arresting the convulsions, as abnormal EEG patterns have been observed after effective magnesium treatment. Alternatively, vasodilatation of the cerebrovasculature may be a factor in seizure arrest. Studies in anaesthetized cats and dogs, and conscious primates (Borges and Gucer, 1978) showed that intravenous  $MgSO_4$  could suppress neuronal burst firing and EEG spike generation induced by topical application of penicillin to the motor cortex. Plasma concentrations of magnesium were below levels that would produce neuromuscular block. The blood brain barrier is

largely impermeable to magnesium, but permeability may be increased under pathological conditions which would allow magnesium administered i.v. to pass into the CSF (Borges and Gucer, 1978). Thus magnesium may act both peripherally and centrally to arrest seizures. The possibility that the anticonvulsant action of magnesium in these circumstances involves an increase or restoration of adenosine action, either directly by enhancing adenosine binding, or indirectly by reducing NMDA channel action, cannot be determined from these studies. However, it is interesting to speculate that adenosine plays a role in the anticonvulsant action of magnesium in the light of the results reported in this thesis.

The interaction of the dihydropyridine calcium channel blockers with the adenosine system appears to occur at both the adenosine receptor and the nucleoside transport system. This results in a differential action between adenosine and the adenosine analogues.

The action of dihydropyridines on the adenosine receptor appears to be exerted by a competitive binding of dihydropyridines to the adenosine receptor. A competitive interaction is supported by the observation that increased concentrations of 2-chloroadenosine were able to reverse the antagonistic action of nifedipine. An alternative possibility is the existence of a distinct low affinity dihydropyridine binding site that allosterically interacts

with the adenosine receptor. Dihydropyridines that bind to this site require a chemical group on position 2 of the phenol ring for effective binding (Fig.3). This is the only consistent difference between the dihydropyridine compounds that interact with adenosine and those that do not. Similarly, verapamil and diltiazem are also unable to interact with adenosine and are presumably unable to bind to the hypothesized low affinity dihydropyridine receptor.

The fact that interactions only occur at concentrations of dihydropyridines much higher than those required for saturated binding, that adenosine has no effect on nitrendipine binding (Morgan et al.,1987) and that, while verapamil and diltiazem can allosterically regulate dihydropyridine binding, they have no effect on adenosine binding (Morgan et al.,1987), strongly suggests that the usual high affinity dihydropyridine binding site is not the site from which adenosine binding is affected. However, the interaction between adenosine and the dihydropyridines indicates a possible association between the two types of receptors. Both receptor types appear to be closely associated with synaptic calcium channels. Murphy et al.(1982) showed that [ $^3\text{H}$ ]nitrendipine binding was concentrated in synaptic regions, particularly in the molecular layer of the hippocampus and the plexiform layer of the olfactory bulb, areas rich in synaptic connections. White matter areas were devoid of binding sites. Similarly, adenosine binding sites are associated primarily with

synaptic regions, particularly in the hippocampus (Tetzlaff et al., 1987).

A study by Thayer et al. (1986) used primary cultures of cells from various regions of rat brain to look at the action of dihydropyridines on potassium stimulated calcium influx as determined by fura 2 microspectrofluorimetry. Interestingly, the inhibition of calcium influx by nitrendipine (1 $\mu$ M) was greater in cultured hippocampal neurones (79% of stimulated influx inhibited) than in striatal neurones (31%), suggesting a differential distribution of dihydropyridine sensitive calcium channels. The distribution of adenosine receptors is also higher in the hippocampus as compared to the striatum (Goodman and Snyder, 1982; Fastbom et al., 1987). In light of the results reported in chapter 2, it is possible that the decreased calcium influx was the result of increased adenosine action via an interaction of endogenous adenosine with the dihydropyridine. This is unlikely for a number of reasons. Endogenous adenosine levels are likely to be low around isolated cultured cells. Nitrendipine was not very effective at decreasing adenosine uptake, as compared to nifedipine and BayK 8644, in the present study. BayK8644 was also used in the experiments by Thayer et al. (1986) producing an increase in calcium influx which would not be expected if an interaction with adenosine was involved. Skattebol and Triggle (1987) also studied the binding of nimodipine and BayK 8644 in different regions of the rat brain. Binding was greatest in the hippocampus and cortex and lowest in the

cerebellum. This is in contrast to adenosine binding where the hippocampus and cerebellum show the highest binding density of adenosine receptors in the brain (Goodman and Snyder, 1982; Fastbom et al., 1987). The dihydropyridine binding distribution was compared with  $^{45}\text{Ca}$  uptake into synaptosomes prepared from the same regions of the brain and was found to be comparable with the predominance of fast (1 sec) uptake components of calcium influx, assumed to be associated with neurotransmitter release, as is the action of adenosine. There is a loose correlation between the distribution of adenosine and dihydropyridine binding sites, with the hippocampus being rich in both types of receptors. However, the cerebellum is particularly rich in adenosine receptors, but comparatively low in dihydropyridine binding sites. A direct comparative study would be needed to determine any real correlation, but at present no direct association between dihydropyridine and adenosine receptors, and subsequently their associated calcium channels, can be drawn.

The main argument against a close association of the two receptors is the functional studies. Adenosine is a potent blocker of synaptic transmission while the dihydropyridines appear to have no effect on calcium channels involved in synaptic transmission. Indeed this is an advantage of the dihydropyridines in the treatment of hypertension and angina as they do not interfere with neuronal transmission (Miller and Freedman, 1984). This is despite the high density of

dihydropyridine binding sites associated with synaptic zones (Murphy et al., 1982) and the fact that, under certain conditions, a block of calcium channels on neuronal cells by the dihydropyridines can be demonstrated (Carboni and Wojcik, 1988; Thayer et al., 1986). Behavioural studies also support the idea that dihydropyridines can affect neuronal activity directly, although it is possible that an interaction with the adenosine system is responsible for some of the behavioural effects of the dihydropyridines.

The dihydropyridines also interact with the nucleoside transport system to reduce adenosine uptake and increase its potency. This is apparent from [ $^3\text{H}$ ]NBI binding studies where the dihydropyridines can potently displace NBI from the nucleoside transport receptor (Morgan et al., 1987). In the present study the addition of dipyridamole effectively removed the interaction of nifedipine with the nucleoside transport system and allowed adenosine to act in a similar manner to the adenosine analogues. The nucleoside transport system is presumably not associated with calcium channels and this interaction demonstrates that the dihydropyridines can bind to sites not associated with the high affinity dihydropyridine binding site. The 2-phenol group dihydropyridines were again more effective than the other dihydropyridine molecules possibly suggesting that a similar low affinity dihydropyridine binding site is responsible for allosteric modulation of both adenosine receptor and nucleoside transport binding sites. However, uptake sites

are not necessarily closely associated with the adenosine receptors, occurring on glial cells (Lewin and Bleck, 1979) as well as neurones, so a direct competitive interaction of dihydropyridine and adenosine at the nucleoside transport site may be a more likely interaction.

The determination of the action of adenosine on calcium influx into synaptosomes demonstrates that adenosine analogues can reduce calcium uptake into the components of a purified synaptosomal fraction of rat brain homogenate. The potassium stimulated component of this uptake is taken to represent uptake into presynaptic synaptosomes across voltage sensitive calcium channels. Contaminants of the synaptosomal preparation probably include glial cells and mitochondria. Stimulated calcium uptake into glial cells has been shown under certain conditions (Hertz et al., 1989), but it is not always apparent (Barnes and Mandel, 1981; Walz and Wilson, 1986). There is no evidence of adenosine affecting calcium sequestration by mitochondria.

The calcium channel at which adenosine might be acting cannot be directly determined using this synaptosomal technique. However, the fact that adenosine action is observed at incubation periods of 20 seconds, but not at 1 second uptake times, may suggest action at a relatively slow inactivating channel such as the novel calcium channel proposed by Suszkiew et al. (1989). The agonist profile

exhibited for the inhibition of calcium influx does not fit either of the established receptor profiles. A proposed  $A_3$  adenosine receptor, possibly linked directly to calcium channels (Ribeiro and Sebastiao, 1986), may present a suitable model for the type of receptor present on the synaptosomes at which adenosine acts.

Unfortunately, the true action of adenosine may have been influenced by the use of potassium to depolarize the synaptosomal membrane together with an influx of calcium via a reversed  $Na^+/Ca^{2+}$  exchange which would reduce the observed reduction in calcium influx by adenosine. Similarly, calcium influx into glial cells cannot be ruled out. If this is the case, the percentage reduction of calcium influx by adenosine is likely to be greater under "normal" physiological conditions. The reduction in calcium influx into hippocampal slices loosely parallels that of the synaptosomes. Inhibition by 2-chloroadenosine was shown to be antagonized by 8-phenyltheophylline, supporting the receptor mediated nature of the inhibition of calcium influx. It is assumed that the majority of calcium influx is into neurones and principally into synaptic terminals. As reported in the introduction to chapter 3, the published reports on the action of adenosine on synaptic calcium influx are largely contradictory. This inability to show a definite action of adenosine is similar to the attempts to determine an action of the dihydropyridines on synaptic calcium influx. Both groups of studies may suffer from the



same technical problems in terms of subgroups of calcium channels not responding to the particular compounds or the true action of the compounds being masked by inactive channels or secondary effects such as reversed  $\text{Na}^+/\text{Ca}^{2+}$  exchange. However, in contrast to the dihydropyridines, adenosine is able to exert a total block on synaptic transmission in electrophysiological studies and, if adenosine acts via calcium channels, the majority of calcium influx into the synaptosomes might be expected to be inhibited. A number of reasons why maximum inhibition by adenosine is not being expressed have been given in the discussion. These conditions would need to be eliminated or controlled to determine the true level of inhibition. What is clear in both the synaptosomes and the hippocampal slices is that adenosine is able to influence calcium influx across voltage sensitive calcium channels. The assumption that this occurs principally into synaptic terminals is based on a large body of experimental evidence from other studies.

The evidence for an action of adenosine on calcium influx is continually growing. Recent evidence includes the experiments of Schubert and colleagues, discussed in chapter 1, where extracellular calcium levels were shown to be reduced upon high frequency stimulation of Schaffer collaterals to CA1 pyramidal cells. This change in extracellular calcium is reduced when adenosine is added indicating that calcium influx is inhibited by adenosine. These experiments are comparable to the synaptosome

experiments in that they study calcium influx across voltage sensitive calcium channels, but there is no potential effect from  $\text{Na}^+/\text{Ca}^{2+}$  exchange. However, influx into glial cells and mitochondrial calcium sequestration cannot be ruled out. Direct recording of synaptic calcium influx is not possible on central neurones. Voltage clamp studies of calcium channels on cultured dorsal root ganglion cells have demonstrated the ability of adenosine to selectively block channels (Dolphin et al., 1986) with the characteristics of the N calcium channel (Gross et al., 1989). This is evidence that adenosine can block calcium channels directly. The possibility that N calcium channels or calcium channels with similar properties, such as the channel proposed by Suszkiew et al. (1989), exist on synaptic nerve terminals is very strong, and it is likely that adenosine will be shown to act on these channels when suitable techniques become available.

### Summary

In conclusion, it is apparent that adenosine compounds can reduce calcium uptake into synaptosomes and hippocampal slices. The synaptosome preparation contains a predominance of presynaptic nerve terminals and it is reasonable to assume that presynaptic calcium influx is being reduced by the action of adenosine. Adenosine appears to act on a voltage sensitive calcium channel as only the potassium depolarized synaptosomes exhibited a reduction in calcium uptake. The nature of the voltage sensitive calcium channel on which adenosine acts is not clear. However, adenosine

does not act on the same channel as the dihydropyridines which have no effect on synaptic transmission or spontaneous activity. The dihydropyridines do, however, influence the action of adenosine and its analogues by interacting at both the adenosine receptor and the adenosine uptake site. This may be via an allosteric interaction from a low affinity dihydropyridine binding site.

Finally, the action of adenosine on synaptic transmission appears to be influenced by activation of the NMDA receptor associated channel. As NMDA channels are normally inactive during glutamatergic neurotransmission this does not affect the action of adenosine. When the voltage sensitive NMDA channels are activated, by removal of magnesium or increased neuronal activation, the ability of adenosine to reduce synaptic transmission appears to be reduced. The mechanism by which this might occur is unclear but it could be of relevance to NMDA related events such as long term potentiation.

#### Future avenues of research.

A number of interesting avenues of research have been opened by the present studies. The nature of the interaction between adenosine and NMDA channels needs to be elucidated, in particular whether the interaction can be isolated to a pre- or postsynaptic site of action.

A possible method of determining whether postsynaptic actions of adenosine are involved in an interaction with NMDA channels would be to carry out the experiments reported

by Lee et al.(1984). Antidromic stimulation of CA1 pyramidal cells in calcium free ACSF results in multiple spikes following the antidromic potential. The magnesium concentration of the ACSF is usually increased to abolish spontaneous activity that interferes with the evoked potentials. The repetitive spikes are inhibited by adenosine and this action is assumed to be postsynaptic, as synaptic transmission is abolished and presynaptic inputs are not being stimulated. It may be possible to study the evoked repetitive spikes in the absence of magnesium and calcium, possibly with an anticonvulsant, such as baclofen, present if necessary. The postsynaptic action of adenosine could then be determined in the absence of magnesium and with increased NMDA channel activation. Attempts were made to study this response but the conditions were not suitable to maintain the multiple spikes, although they were apparent briefly. These responses are very susceptible to changes in flow rate and temperature (Fowler, 1988). These experiments will be pursued at a later date when the ideal conditions have been determined.

An effect, that may or may not be related to NMDA channel activation, was observed during preliminary HPLC analysis of amino acid release from the slices during these experiments. The release of aspartate from the slice increased during perfusion with adenosine in magnesium free ACSF. This was in contrast to control magnesium ACSF where aspartate release was largely unaffected. Glutamate release did not appear to

be affected by adenosine. The possibility arises that the loss of adenosine inhibition in magnesium free ACSF is due in part to the increased release of aspartate, which may act at the NMDA receptor. This may contribute to the reduced inhibitory action of adenosine by actively increasing the excitability of the cells when adenosine is added. It may also explain increases in potential size seen at low adenosine concentrations in magnesium free ACSF (Fig.1.4.). These observations have not been included in the results as the analysis was carried out on only 3 slices with large variations in amino acid release between the slices. Consequently, the increased aspartate release cannot be confirmed as a genuine response to adenosine. This does, however, provide another avenue to explore in future studies on the effects of adenosine.

Other lines of research include a study of other brain regions to determine whether an interaction between adenosine and NMDA channels is widespread or confined to the hippocampus and dentate gyrus and elucidation of the mechanisms by which an interaction may occur, including the involvement of protein kinase C and the proposed retrograde messenger, arachidonic acid.

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